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USE OF SUPERCRITICAL CARBON DIOXIDE FOR EDIBLE OIL PROCESSING

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled USE OF SUPERCRITICAL CARBON DIOXIDE FOR EDIBLE OIL PROCESSING submitted by Nurhan Turgut Dunford in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Processing/Engineering.

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ABSTRACT

The main objective of this thesis work was to investigate the various processing parameters, such as temperature, pressure, cosolvent (ethanol, EtOH) addition and moisture content of feed material on lipid extract composition, residual proteins and enzyme activity during supercritical (SC) CO_2 extraction of canola and Atlantic mackerel (*Scomber scombrus*), representing low and high moisture products, respectively.

Phospholipid (PL) recovery of 20.8% was achieved when canola flakes were extracted at 70°C and 55.2 MPa with SC-CO_2/10% EtOH following oil removal with EtOH-free SC-CO_2. Soaking of canola meal with ethanol prior to SC-CO_2/EtOH extraction increased PL recovery to 30.4%. Fractionation of acetone insoluble gums resulted in extracts containing 50% PL, of which 90% was phosphatidyl choline, but yields were very low due to caking.

Amount of SC-CO_2 extract from preheated and cooked canola flakes containing 12.7-42.5% (w/w, dry and oil-free basis) moisture levels increased with temperature and pressure, except at 20.7 MPa it decreased with temperature due to crossover of solubility isotherms. Some water was co-extracted with canola oil. Free fatty acid (FFA) concentration of the extracts increased with decreasing pressure. The cooking process improved extract yield only slightly while resulting in higher FFA levels in SC-CO_2 extracts compared to extracts of the preheated samples.

Investigation of the myrosinase activity and glucosinolate hydrolysis during SC-CO_2 extraction of flaked and whole canola seeds showed that combined effects of high temperature (75°C), pressure (62.1 MPa) and moisture (20%) were necessary to achieve

90% reduction in enzyme activity. Glucosinolate degradation was minimal at low moisture levels. Myrosinase inactivation in whole seeds was more difficult than in flakes.

Freeze-drying of Atlantic mackerel muscle down to 10.2% (w/w) was sufficient for high oil yield and minimum changes in proteins. Aggregation of low molecular weight sarcoplasmic proteins to form larger proteins was observed during SC-CO_2 extraction of mackerel at all moisture levels studied. SC-CO_2 extracted fish muscle had lighter color than feed material.

These studies contribute to our limited understanding of the complex interactions between SC-CO_2/water/oil/cosolvent/solid matrix components during high pressure extraction.

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NOMENCLATURE

A_B	Mean absorbance of blanks
AI	Acetone insoluble fraction of crude lecithin
A_S	Mean absorbance of standards
AOCS	American Oil Chemists Society
a_w	Water activity
A_X	Absorbance of sample
BET	Brunauer-Emmett-Teller (equation)
C	Constant
CE	Capillary electrophoresis
CO_2	Carbon dioxide
C_S	Concentration of standards, µmol/mL
C_X	Concentration of glucosinolates in sample, µmol/g
DEAE	Diethylaminoethyl
D_F	Dilution factor
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EtOH	Ethanol
FFA	Free fatty acids
FDA	Food and Drug Administration

GC	Gas chromatograph
GRAS	Generally recognized as safe
K	Absorption factor
LSD	Least significant difference
MPa	Mega pascal
O/W	Oil-in-water emulsion
PA	Phosphatidic acid
PC	Phosphatidylcholine
P_c	Critical pressure
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipids
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
SAS	Statistical analysis systems
SCF	Supercritical fluid
SCFE	Supercritical fluid extraction
SDS	Sodium dodecyl sulphate
T_c	Critical temperature
TG	Triglycerides
UV	Ultraviolet detector
W	Weight of sample, g

W/O	water-in-oil emulsion
X	Equilibrium moisture content, g water/g dry solid
X_m	Monolayer moisture content, g water/g dry solid

1. GENERAL INTRODUCTION

Fats are one of the three major classes of food components, others being the proteins and carbohydrates. The oldest known fats are those found in pots in the tombs of pharaohs (Hoffmann, 1989). Mediterranean olives, rapeseed from Europe, sesame seed from India and soybeans from China are the oldest oil-rich crops used by the ancients.

Edible oils and fats are essential nutrients of the human diet. They are the most concentrated source of food energy. Edible oils and fats supply about 9 Kcal/g of energy compared to about 4 Kcal/g from proteins and carbohydrates. Fats and oils provide 40% of the caloric needs of people living in North America (Vaisey-Genser and Eskin, 1987), even though it is recommmended to be 30% (Health and Welfare Canada, 1990). Fats are the major sources of fat soluble vitamins A, D, E, and K. Ingestion of fat also improves the absorption of these vitamins. Fats are vital in the human diet for providing the essential fatty acids, linoleic and linolenic. Fats are also essential components of all cell membranes. They serve as thermal insulation for the body and protect internal organs against external shocks. Lipids have important functions in biochemical regulatory systems, e.g. prostaglandins.

Growing public health and fitness awareness and advances in nutritional research have raised the debate on the pros and cons of the various dietary fats. Today, most of the available data related to the effects of fats on human health seem to be contradictory and confusing for the public. Effect of various edible fats on growth rates, blood cholesterol levels and development of cancer, immune system disorders and cardiovascular diseases needs to be clarified by further research.

Fats play a very important role in food processing. Frying fats and cooking oils control heat exchange in the medium and they contribute to color and flavor as well. Shortenings contribute to tenderness of baked goods. Salad oils carry flavors and contribute to mouthfeel. Mono- and diglycerides and some of the phospholipids function as emulsifiers.

1.1. CHEMISTRY OF LIPIDS

It is not possible to define lipids in simple chemical structural terms. However, lipids are those components that can be extracted with organic solvents from plant and animal matter. Carbohydrates, proteins, ions and very polar compounds do not dissolve in organic solvents. Lipids are broadly classified into three groups (Bloor, 1925);

1) Simple lipids (Neutral lipids):

    a) Acylglycerols or fats: esters of fatty acids with glycerol;

    b) Waxes: esters of long-chain fatty acids with long-chain alcohols other than glycerol;

2) Compound lipids: compounds containing other groups in addition to an ester of a fatty acid with an alcohol;

a) Phospholipids (Phosphatides): esters containing fatty acids, phosphoric acid, and other groups usually containing nitrogen;

b) Glycolipids (Cerebrosides): compounds containing fatty acids, a carbohydrate and a nitrogen moiety, but no phosphoric acid;

c) Other compound lipids: sphingolipids and sulfolipids; and

3) Derived lipids: compounds derived from neutral or compound lipids and having general properties of lipids: e.g. fatty acids, sterols.

The term "fat" is used for all triglycerides regardless of whether they are liquid or not at room temperature. Oils are usually liquid at ambient temperature. Edible oils and fats are composed primarily of triglycerides (TG) which are the esters of one molecule of glycerol and three molecules of fatty acids (Fig. 1.1). If only one or two fatty acids are attached to a glycerol molecule, this molecule is called mono- or diglyceride, respectively. Fatty acids which are not linked to the glycerol backbone or some other molecule in a fat are referred to as free fatty acids (FFA). According to the Geneva system of nomenclature, a Greek prefix indicates the number of carbon atoms in a fatty acid chain. The carbons in a fatty acid chain are numbered from the carboxyl end of the carbon chain and the suffix "anoic" and "enoic" are used for saturated and unsaturated bonds, respectively. For example, according to the Geneva system, oleic acid with 18 carbon atoms and one double bond is named as octadecenoic acid. The other system used by biochemists to designate the unsaturated fatty acids is the "omega" or "n" classification. The term "omega (ω)" refers to the position of the first double bond from the methyl end of the molecule (e.g. linolenic acid is an ω-3 (n-3) fatty acid). Fatty acids found in foods, with a few exceptions, are straight chain molecules with an even number of carbon atoms. C18 acids, stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3), and C16 acids, palmitic (C16:0) and palmitoleic (C16:1) acids are found in most food fats. Long and branched-chain fatty acids and those with acetylenic (triple bond) groups occur in plants and microorganisms.

Phospholipids (PL), together with proteins and glycolipids, are the main components of biological membrane structures. They invariably occur in all foods of plant and animal origin. The chemical structure of PL is similar to that of triglycerides, except one of the fatty acids is replaced with phosphoric acid and an N-containing group. Phosphatidyl choline or lecithin (PC), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), and phosphatidyl inositol (PI) are the main phospholipid derivatives associated with dietary fats. The chemical structures of phospholipid derivatives are given in Figure 1.2.

Phospholipids are soluble in alcohol, benzene, ether and chloroform but are practically insoluble in acetone, ethyl acetate and similar esters. Phospholipids are not water soluble but they form colloidal dispersions in aqueous media and act as emulsifying agents. An emulsifier is a substance with a hydrophobic and a hydrophilic group on the same molecule and thus, has the capability of forming water-in-oil (W/O) or oil-in-water (O/W) emulsions by reducing the interfacial tension between the two phases. Crude industrial lecithin, which is a mixture of PC, PE and PS, has poor W/O and O/W emulsifying properties, because the PC favors O/W emulsification whereas PE promotes W/O emulsions.

Table 1.1 shows total lipid and PL contents of various oilseeds. Phosphatide content of seeds depends on the particular species of plant, maturity of the seed when the oil is extracted, climate and nature of the soil. Choice of solvent and method of extraction also affect the PL content of the extracted oil. PC and PE contents of phospholipids vary depending on the type of oilseeds as given in Table 1.2.

1.2. SOURCES AND NUTRITIONAL ASPECTS OF EDIBLE FATS

Fats can be of animal or vegetable origin. Total annual world oil and fat production is about 84 million metric tons (Anon., 1993a). Fats of vegetable origin have the highest share (80%) in world fat production (Lawson, 1995). About a hundred plant varieties are known to have sufficient oil content for commercial production. However, only 22 types of vegetable oils are produced commercially. Oilseeds and their products are the most valuable agricultural commodity in the world trade (Shahidi, 1990). Table 1.3 shows annual world production, consumption and export of 10 major fats and oils. Although world animal fat consumption has continued to decline over the last century, it is still the second largest fat group produced worldwide. Oil from marine sources makes up about 2% of world production (Bimbo, 1990).

Currently, oilseed related scientific research is focused on four areas: a) increasing the oil content of the seed (Arthur and Ford, 1995; Wratten and Mailer, 1995), b) increasing oil yield/unit area of land (Downey and Röbbelen, 1989), c) modifying the fat composition (Möllers and Albrecht, 1995), and d) developing disease and pest resistant varieties (Grezes-Besset et al., 1995). Some of the new crops which have been studied for their utilization in food and oleochemical industries are jojoba, evening primrose, borage and blackcurrant seed (Gunstone, 1989). Recently, the use of refined low-linolenic flax oil in food applications was approved by Health and Welfare Canada (Anon., 1993b).

1.2.1. Canola

In Canada, the name "canola" was adopted in 1979, for plant varieties genetically engineered from rapeseed which is a *Brassica* oilseed. The origin and description of the *Brassica* oilseed cultivar were reviewed by Downey (1983). Currently, canola refers to a rapeseed cultivar that contains <2% erucic acid (C22:1) in its oil and <30 μmol/g of one or any combination of the four known aliphatic glucosinolates (gluconapin, progoitrin, glucobrassicanapin and napoleiferin) in its defatted meal (Shahidi, 1990). Summer rape (*Brassica napus*) and summer turnip rape (*Brassica campestris*) varieties are grown in Canada. In 1985, the U.S. Food and Drug Administration (FDA) granted GRAS (generally recognized as safe) status to canola. In Canada, annual canola production is about 4 million metric tons (Shahidi, 1990). Of this total, 40% has been processed domestically (Shahidi, 1990). Canada is the second largest canola seed exporting country in the world after Germany.

Oil content of canola seed is 40-44% (w/w), which is higher than that of soybean (18-20%). Canola oil has a low level of saturated fatty acids (~6%), a relatively high level of monounsaturated fatty acid, (oleic acid, ~60%), and an intermediate level of the polyunsaturated fatty acids (PUFA) (linoleic and linolenic acids, ~20 and ~10%, respectively). Canada permits only 2% erucic acid in canola oil, while up to 5% is allowed according to the Codex Alimentarius standard (Vaisey-Genser and Eskin, 1987). Fatty acid composition of selected oils are given in Table 1.4.

Vegetable oils are the major sources of vitamin E in the diet. Tocopherols occur in several forms which vary in biological activity. Although total tocopherol content of canola oil (66 mg/100 g) is less than that of soybean oil (104 mg/100 g), α-tocopherol which has vitamin E activity, is higher (19 mg and 10 mg/100 g, respectively) (McDonald, 1987). Sterol content of canola oil was examined by Ackman and Sebedio (1981). Brassicasterol (7.4 mg/100 g), campesterol (35.5 mg/100 g) and β-sitosterol (57.1 mg/100 g) were found to be the major sterols in canola oil and it was indicated that sterol level and types were nutritionally useful and nontoxic in all respects. Subbiah (1973) reported that plant sterols, particularly β-sitosterol, were effective in lowering serum cholesterol through their effect on the absorption of cholesterol from the intestine. Sulphur compounds, which cause catalyst poisoning during the hydrogenation process, have not been effectively eliminated by the reduction of glucosinolates in the rapeseed. Due to its relatively higher sulphur content (<3 ppm), canola oil can be hydrogenated with only 50-80% of the efficiency of soybean oil (Anon., 1981).

Protein content of canola meal is about 36-37%. Although canola meal is a good source of high quality protein, its use for human food and animal feed is limited due to the presence of high fibre and antinutritional components such as glucosinolates, polyphenols and phytic acid. Phytic acid can bind with minerals and proteins to reduce their bioavailability. Alli and Houde (1986) studied the phytates of canola in detail. Phenolic compounds may contribute to the dark color, bitter taste and astringency of meals. Kozlowska et al. (1990) reviewed the phenolic compounds in canola. Although glucosinolates are relatively nontoxic, their hydrolytic products are undesirable goitrogenic aglucones with toxic effects. Types and levels of glucosinolates in canola have been studied by several researchers (Slinger, 1977; Daun, 1986; Shahidi and Gabon, 1989; Campbell and Slominski, 1990).

It was shown that canola oil was effective in lowering serum cholesterol levels in human subjects (Bruce and McDonald, 1977; Bruce et al., 1980; McDonald, 1983), which is most probably due to the cholesterol lowering effect of oleic acid (Mattson and Grundy, 1985; Grundy, 1986). Although erucic acid is well digested by humans (Vaisey-Genser et al., 1973), its significance in the human diet is not well defined. Erucic acid was related to fat accumulation in the heart muscle of male rats (Abdellatif and Vles, 1970; Beare-Rogers, 1970); however, studies with other species have failed to show clear evidence of erucic acid involvement in cardiovascular diseases after long term feeding of canola oil (McCutcheon et al., 1976).

1.2.2. Fish

About 1.5 million metric tons of fish and marine oils are produced worldwide (Bimbo, 1990). In Europe, they are used in food formulations such as margarine, shortening, cooking and salad oils. The first commercial spread using refined and deodorized fish oil was made in Denmark (Haumann, 1990). Fish oils are also used in animal feed, food supplements, paints and in a variety of other industries. FDA affirmed GRAS status in United States for partially and fully hydrogenated menhaden oils in foods in 1990. The second part of the GRAS petition covering refined and deodorized menhaden oil and the GRAS status for other fish oils are expected to be approved in a few years (Haumann, 1990). In Canada, although fish oils were historically used in lower priced margarines, they no longer are, due to the decision of the food manufacturers to label their products "all vegetable" in 1985 (Haumann, 1990).

The fat content of fish ranges from 0.5 to 24% (Kinsella, 1990). Lipid composition of fish oil is similar to that of vegetable oils consisting of a mixture of triglycerides with small amounts of mono- and diglycerides, phospholipids, FFA and sterols. Typical fish oil fatty acid composition is given in Table 1.4. The saturated fatty acids in fish oils have carbon chain lengths that generally range from C12 (lauric acid) to C24 (lignoceric acid). Carbon chain lengths of the unsaturated acids range generally from C14 (9-tetradecenoic acid) to C22 (4,7,10,13,16,19-docosahexaenoic acid). The polyunsaturated ω-3 fatty acids, linolenic (C18:3), eicosapentaenoic (EPA, C20:5) and docosahexaenoic (DHA, C22:6) acids are essential for biological development. Principal marine oil polyunsaturated fatty acids are in the ω-3 category. The EPA and DHA content of fish oil varies from 5 to 24% and 4 to 37%, respectively, of total fatty acids (Kinsella, 1986). Current interest in fish oils is due to their long chain ω-3 fatty acids which are reported to have therapeutic effects on human health (Ackman, 1988). Omega-3 fatty acid concentrates are available in the market and their consumption was projected to increase in the future (Ackman, 1989).

Atlantic mackerel (*Scomber scombrus L.*) is a Canadian pelagic fish species. Although it has been a delicacy in Europe, mackerel is underutilized in North America. Canada has one of the largest mackerel stocks in the world (Department of Fisheries and Oceans, 1991). Its high lipid (1.8-20.6%) (Ackman, 1989) and PUFA (30%) (Ackman and Eaton, 1971) contents make Atlantic mackerel a perishable item. Thus, its postharvest handling and processing pose challenges for maintenance of good quality.

Even though the lipid content of Atlantic mackerel exhibits substantial seasonal variation (1.8% in the spring vs. 20.6% in the late fall), its protein content is relatively constant throughout the year at 17.4% (Leu et al., 1981). Muscle proteins can be classified into three main groups based on their solubility characteristics: a) sarcoplasmic (myogen): soluble in water or dilute salt solutions, b) myofibrillar: soluble in concentrated salt solutions, and c) connective tissue proteins: insoluble in concentrated salt solutions (Lawrie, 1974). The sarcoplasmic proteins are located in the intracellular fluid called sarcoplasm. This fraction includes a diverse group of proteins (100-200 proteins), including most of the enzymes involved in metabolism and they comprise about 30-35% of the total muscle protein (Pearson and Young, 1989). Sarcoplasmic proteins are also heavily involved in discoloration problems of fish products (Jones, 1962).

1.3. EDIBLE FAT EXTRACTION TECHNOLOGY

1.3.1. Conventional extraction

Extraction is a separation process which involves dissolution of the desired components of a mixture of substances into a solvent. The theory of "selective wetting of surfaces" (Rehbinder, 1933) is a common feature of all fat separation processes. This theory is based on the displacement of fat from its high-moisture surroundings by water at a high temperature. When two immiscible liquids compete for wetting of the same surface, the one having the lower surface tension will preferentially wet it. At an optimum temperature, polar water will selectively wet protein/carbohydrate structures breaking the weak bond between them and nonpolar fat molecules. Thus, water displaces the fat which coalesces to form a continuous fat phase. Then, fat is mechanically expressed out of the tissue and/or dissolved into a nonpolar solvent. Although general principles of all fat separation processes are common, structural differences in animal and plant fatty tissues affect the degree of processing during fat extraction. Animal fats are generally located in the white adipose tissue cells as one single globule (unilocular cell). These cells do not have a cell wall and the cell membrane is composed of glyco- and phospholipids. Marine animals, like whales, sea elephants and seals, contain up to

70% fat in their adipose tissue, whereas oily fish (anchovy, herring, menhaden, mackerel) may contain about 10-20% fat in the whole body (Hoffmann, 1989). The fat in plant cells is generally located in the peripheral parts of the cytoplasm as small globules. Besides a cell membrane, plant cells also have a cellulose and hemicellulose based cell wall.

1.3.1.1. Oilseeds

Edible oils are extracted from oilseeds conventionally, with hexane in a semi-continuous countercurrent process. An oilseed processing system consists of the following steps (Allen et al., 1979; Hoffmann, 1989):

a) Flaking: The rate controlling step in solvent extraction of seeds is the molecular diffusion of solvent and oil in the particle due to the internal resistance of the seeds. Reduction of particle size minimizes such internal resistance. As well, flaking of oilseeds creates a large surface to volume ratio resulting in better contact between solvent and feed.

b) Cooking: Changes brought about by cooking are both chemical and physicochemical in nature, such as protein denaturation to release oil from the cell structure and inactivation of enzymes. Also, cooking process gives seeds proper plasticity for efficient pressing. Canola is preheated to 20-50°C in <5 min and cooked at 80-90°C for 15-30 min to condition the seeds for improved oil extraction and to inactivate the endogenous enzyme, myrosinase.

c) Mechanical expression of oil: In this step, oil content of the canola seeds is reduced to 15-18% from 40-44%. Seeds with a low oil content like soybean are hexane extracted without pressing.

d) Solvent extraction: Press cake is extracted with hexane at 50-60°C in shallow (50 cm) or deep-bed (120-210 cm) extractors. Flow rate of hexane to the extractor is adjusted to provide a micella (hexane-oil mixture) concentration of 25-33% oil at the extractor outlet. Oil content of the meal remaining after solvent extraction is usually 1-3%.

e) Desolventizing: Evaporation of solvent from the extracted cake is achieved in a desolventizer-toaster vessel.

f) Distillation: Solvent is recovered from oil by distillation. First stage distillation concentrates the micella from 25-33% to 65% oil in hexane. In the second stage, micella is further concentrated to 95%. The final stripping stage reduces the hexane concentration in the oil to <500 ppm. Crude canola oil contains phosphatides, insoluble matter, free fatty acids, pheophytin, colored bodies and other minor impurities.

g) Degumming: Phospholipids are removed from the oil to avoid their precipitation during storage. Water (80°C) or steam is used to reduce the phosphatide content of the oil down to ~100 ppm. Phospholipid content of the oil can be further reduced to ~50 ppm by super-degumming with the addition of 1500-2500 ppm aqueous organic acid into the oil at 40-55°C. After centrifugation and drying to remove free moisture, the oil is transferred to the refining process.

h) Refining: There are two methods to refine edible oils, alkali and physical refining. In the case of alkali refining, heavy soap stock formed by addition of aqueous sodium hydroxide is separated from crude oil by gravity settling or centrifugation. Physical refining uses high temperature distillation at low pressures for the removal of free fatty acids from the oil.

i) Bleaching: This is carried out with acid-activated clay to remove carotenoid and chlorophyll pigments.

j) Deodorization: It is performed by steam stripping. When physical refining is used, the deodorization step is combined with the refining process.

h) Hydrogenation: Hydrogenation of the refined oil is done by saturating the double bonds of the carbon chains of fatty acids in the triglycerides with hydrogen gas over metal catalysts (usually nickel).

1.3.1.2. Lecithin

Phosphatides or lecithin separated from crude canola oil are not utilized for food purposes, but are added back to the meal to enhance its feeding value. On the other hand, soybean lecithin is further refined for various applications in the food, feed and pharmaceutical industries as emulsifiers. Industrial scale modification of crude lecithin is achieved by physical, chemical and enzymatic means (Van Nieuwenhuyzen, 1976).

1)Physical modification:

a) Acetone extraction: Commercial oil removal from crude lecithin is performed with warm acetone. After drying, the end product is a waxy solid which can be formed into a specific particle size.

b) Alcohol fractionation: The principle of this process is based on the differences in the relative solubilities of PL and PC in alcohols. Since PC dissolves more readily in ethanol than PE, products with a PC:PE ratio of >8:1 can be obtained by using 90% ethanol. Varying processing parameters such as alcohol polarity, concentration, lecithin/alcohol ratio, temperature and extraction time, can result in products with varying PC/PE ratios.

c) Spray drying: Lecithin is spray dried onto proteins which act as a carrier. By this method, it is possible to obtain products in powder form containing 30-40% lecithin at a relatively low cost.

d) Spray cooling with synthetic emulsifiers: Combination of 30% lecithin and 70% mono- and diglycerides by spray cooling results in flaked or powdered products with improved emulsifying properties.

2) Chemical modification:

a) Acetylation: Treatment of raw lecithin with acetic anhydride acetylates the amino group of PE. The principle of this process is to block the "zwitter-ion" group of the PE improving the O/W emulsifying property of crude lecithin.

b) Hydroxylation: High concentrations of hydrogen peroxides are added with acids, especially lactic acid, to form hydroxyl groups in the unsaturated fatty acid chains. The end product has improved O/W properties and it is easily dispersable in cold water. Application of these products in the food systems has been legally limited in many countries.

c) Hydrolysis by acids and bases: Hydrolysis of fatty acids under acidic or basic conditions usually leads to lecithins with undesirable dark color.

3) Enzymatic modification:

Lecithin with more hydrophilic and stronger O/W emulsifying properties and reduced Ca sensitivity is produced by using the enzyme phospholipase A2 which hydrolyzes the fatty acid at the β-position of the lecithin molecule.

Several of the above mentioned processes can be combined to obtain a custom designed lecithin product for specific product applications.

1.3.1.3. Fish oils

Commercial fish meal and oil are produced by a wet rendering process which separates liquid from solids by use of water or steam. The fish oil production process includes cooking, pressing, drying, separation and evaporation steps (Hoffmann, 1989). Cooking coagulates the fish proteins. The oil and water are separated from solids by pressing. Drying reduces the moisture in the press cake for longer storage. Oil and water mixture is then centrifuged for oil separation. Aqueous (stickwater) phase is concentrated in continuous vacuum evaporators from 6% to 50% solids content. The concentrate is called "fish solubles" which is usually added back to press cake or stabilized with acid and sold as an ingredient for feeds, fertilizer or fermentation. The oil fraction is washed, centrifuged and stored as crude fish oil. Refined fish oil is obtained after alkali refining, winterization and bleaching.

Due to the increasing interest in the ω-3 fatty acids, fish oils are further fractionated to concentrate these nutritionally beneficial components. Conventional techniques used to concentrate ω-3 fatty acids are crystallization, fractional or molecular distillation and adsorption chromatography (Mishra et al., 1993). Fish oil triglycerides are hydrolyzed to their constituent fatty acids and then converted to their ethyl or methyl esters prior to the use of these fractionation techniques which exploit the differences in chain length and degree of unsaturation between the fatty acids and their esters.

1.3.2. Supercritical fluid extraction

Although Baron Cagniard de La Tour discovered the critical point of a substance in 1822, the ability of a supercritical fluid (SCF) to dissolve low vapour-pressure solid materials was first reported by Hannay and Hogart in 1879 (McHugh and Krukonis, 1994). They observed the solubilization of inorganic salts by changing the pressure of gaseous ethanol at its critical temperature of 234°C (McHugh and Krukonis, 1994).

1.3.2.1. General principles

Although matter is usually classified into three phases, namely gas, liquid and solid, it enters a fourth phase which is referred to as the "supercritical phase" when it is compressed to a critical pressure (P_c) and heated to a critical temperature (T_c) (Fig.

1.3). Matter which is in the supercritical region is described as a SCF. Table 1.5 shows the P_c and T_c of selected compounds (Weast, 1977-78). An important criterion for solvent selection for supercritical fluid extraction (SCFE) processes is the critical point of the solvent. Choosing a solvent with a high P_c will result in an expensive process because of high equipment and maintenance costs at high pressures. If T_c is too high, heat sensitive materials will be destroyed during the process. This is very critical for applications of SCFE in the food industry, since excessive heat treatment causes loss of flavor, texture and nutritional value of foods. Carbon dioxide (CO_2) is an ideal solvent for food applications of SCFE because it is readily available, cheap, non-toxic, non-corrosive, non-explosive and easily removable from the extracted product. Most importantly, it has moderate P_c and T_c values of 7.3 MPa and 31°C, respectively.

Physical properties of a SCF fall between those of a liquid and a gas. Supercritical fluids have higher diffusivities than liquids; hence they move through the material to be extracted much faster than liquids. The pumping cost of SCF's is lower than that of liquids because of their lower viscosities. The densities of SCF's are similar to those of a liquid; thus, they have the solvent power of conventional organic solvents. Furthermore, a unique feature of the SCF's is the adjustability of their density by changing temperature and pressure, especially near the critical point (Fig. 1.4). Principles of SCFE technology were reviewed in several publications (Irani and Funk, 1977; Schneider, 1978; Peter and Brunner, 1978; Brunner and Peter, 1982a; Rowlinson, 1983; Rizvi et al., 1986; McHugh and Krukonis, 1994). SCFE has several attractive features:

a) Thermally labile and/or high-boiling point components of biological matters can be extracted at relatively low temperatures which are usually close to the critical temperature of solvents.

b) SCF's can dissolve non-volatile substances.

c) Compounds can be selectively extracted by changing the density of the SCF.

d) Product recovery is quite easy, since relatively small changes in temperature and pressure of the extraction medium can result in considerable changes in solubility. This feature of SCFE is unique and a distinct advantage over liquid extraction where solvent separation is costly and most of the time is incomplete, furthermore it requires heat treatment of the product.

e) If necessary, the solvent power of a SCF may be enhanced by the addition of a cosolvent (entrainer).

f) Faster mass transfer rates can be achieved with SCF's due to their lower viscosity and higher diffusivity than liquids for a given density.

1.3.2.2. Food applications of SCFE

In the early 1900's, the most extensively studied system was the solubility of naphthalene in various SCF's (Diepen and Scheffer, 1948, 1953). The SOLEXOL process was developed for the purification and separation of vegetable and fish oils (Dickinson and Meyers, 1952). In this process, near-critical propane was used to fractionate crude fish oil into a vitamin-A concentrate, fatty acid fractions with low and high unsaturation levels, color compounds and "insolubles" (Passino, 1949; Dickinson and Meyers, 1952). In the early 1980's, research and development in SCFE were mainly on the extraction of commodity chemicals and synthetic fuels. Over the last decade, use of SCF's has become more interdisciplinary, leading to new applications in extraction, fractionation, chromatography, nucleation, and chemical reaction processes. Several food applications have reached commercial level. Industrial scale SCFE plants processing coffee, tea, hops, spices and flavors have been in operation since mid 1985 in the United States, Germany, France, United Kingdom and Japan (McHugh and Krukonis, 1994). Recently, a low-fat, low-cholesterol egg yolk product extracted with supercritical carbon dioxide (SC-CO_2) was introduced into the market in the U.S. (Bringe and Cheng, 1995).

1.3.2.2.1. Cholesterol and fat reduction

Growing consumer concern over dietary fat intake has accelerated the research related to fat, cholesterol and saturated fat reduction in food products. SC-CO_2 extraction technology provides an alternative method for fat removal from biological systems without any organic solvent residue as well as minimal denaturation of proteins and loss of functional properties. Wong and Johnston (1986) have studied the solubility of various sterols in SC-CO_2. Extraction of cholesterol from egg yolk (Leiner, 1986; Froning et al., 1990; Bulley et al., 1992; Bringe and Cheng, 1995), milk fat (Arul et al., 1988; Bradley, 1989; Lim et al., 1991), beef (Chao et al., 1991; Wehling et al., 1992; King et al., 1993) and fish muscle (Hardardottir and Kinsella, 1988) were achieved using SC-CO_2. Solubility data and phase behaviour for pure fatty acids, fatty acid methyl and ethyl esters, mono-, di- and triglycerides in SC-CO_2 have been reported by numerous researchers since 1978 and were reviewed by Hammam (1994). Fat extraction from different meat products with SC-CO_2 has also been studied by various research groups. Bone meal (Zosel, 1982), link sausage, luncheon meat and ham (King et al., 1989), ground beef (Chao et al., 1991), dehydrated beef powder and chunks (Wehling et al., 1992), beef patties (King et al., 1993) and chicken meat (Froning et al., 1994) were some of the meat products investigated.

1.3.2.1.2. Oilseeds

Some of the oilseeds successfully extracted with SC-CO_2 are soybean (Stahl et al., 1983; Friedrich, 1984; Snyder et al., 1984), jojoba (Stahl et al., 1983), cottonseed and corn (Friedrich and Pryde, 1984), sunflower and rapeseed (Stahl et al., 1980), palm (Kalra et al., 1987), evening primrose (Favati et al., 1991), wheat germ (Taniguchi et al., 1985), gluten (Hammam et al., 1987; Hammam and Sivik, 1991) and canola (Bulley et al., 1984; Fattori et al., 1987, 1988; Temelli, 1992). These studies showed that oil solubility in a SCF is strongly correlated with solvent density. The refining costs for SCF extracted oil were reduced because phospholipids and glycolipids are extracted only in trace amounts or not at all. Fatty acid composition of the SC-CO_2 extracts obtained at 34.5-62.1 MPa and 35-70°C was similar to that of hexane extracted oils (Table 1.6). During semi-batch oilseed extraction, smaller molecular weight compounds, such as FFA and odor compounds, are extracted more at the beginning, whereas pigments, other nonsaponifiables and PL are removed towards the end of the process. Overall FFA and nonsaponifiables contents and peroxide value of SC-CO_2 extracted oils are the same order of magnitude as those for the hexane extracts. Heavy metal, especially iron, contents of the hexane extracted oils are higher than those of the SCFE extracts.

The first report on SC-CO_2 extraction of canola was published by Bulley et al. (1984). In this study, basic equations governing the oil extraction from a bed of seeds were derived. Also, canola oil solubility at 30-35 MPa, 40°C and three different CO_2 flow rates (0.45, 0.64 and 1.84 g CO_2/min) were determined. It was shown that oil solubility and extraction rates increased with pressure and CO_2 flow rate, respectively. Later, Fattori and coworkers (1988) studied the effect of extraction conditions and seed treatment on the canola oil solubility in SC-CO_2. They achieved highest oil solubility, 11 mg/g CO_2, at 36 MPa and 55°C. Among the different seed pretreatments studied (flaking, cooking, pressure rupturing, chopping and crushing), cooked and flaked seeds gave the maximum yield. Phosphorus content and fatty acid composition of canola oil extracted at 36 MPa and 55°C with SC-CO_2 were determined (Fattori et al., 1987). Phosphorus content of SC-CO_2 extracts was less than 7 ppm. It was shown that only small variations occurred in the fatty acid composition of canola oil throughout the SC-CO_2 extraction; however, the last 20% of the oil had longer chain fatty acids (C20-24) in larger proportion than the previous fractions. Temelli (1992) studied extraction of TG and PL from canola with SC-CO_2 and ethanol (EtOH) and demonstrated that SC-CO_2/EtOH extracts contained PL. Concentration of longer chain fatty acids (C20-22) was higher in the SC-CO_2/EtOH extracts compared to the EtOH-free SC-CO_2 extracts.

Crude lecithin was another raw material investigated for oil removal with SC-CO_2. Heigel and Hueschens (1983) patented a SC-CO_2 extraction process involving the production of de-oiled lecithin from crude soybean lecithin containing 30% oil. Castera (1994) extracted commercial liquid lecithin with SC-CO_2. The lecithin obtained with SC-CO_2 was darker in color but had less odor than the products obtained by acetone treatment. Alkio et al. (1991) have developed a process to de-oil crude oat lecithin with SC-CO_2. A continuous near-critical fluid extraction process which utilized a mixture of CO_2 and propane as a solvent for the production of de-oiled lecithin was developed by Peter et al. (1985). Their end product was a tasteless, yellowish-white powder and free of solvent. SC-CO_2 exhibits a low solubility for the relatively polar PL. To date, the studies on oil extraction from oilseeds have established that EtOH-free SC-CO_2 does not extract PL from crushed oil seeds (Friedrich and Pryde, 1984; Fattori et al., 1987). However, the addition of a polar cosolvent would enhance the selectivity of the supercritical solvent towards the PL. Recovery of PL from canola flakes and press cake (Temelli, 1992), freeze-dried egg yolk (Bulley et al., 1992), cottonseed (Sivik et al., 1994) and soybean flakes (Montanari et al., 1994) was investigated.

1.3.2.2.3. Cosolvent effects

Addition of a cosolvent (also called entrainer, modifier, moderator) to a mixture containing a supercritical component can modify the extraction selectivity markedly. Cosolvents, which are mostly liquids and have solubility parameters larger than that of the extracting agent, can be used to raise the solvent power of a SCF while maintaining adjustability of its density. They also increase yields, or decrease pressure and solvent requirements of the SCFE. Cosolvents can affect the system by increasing volatility of the solutes, by increasing the density of the SCF or by enhancing the miscibility. Hexane, benzene, chloroform, isopropanol, methanol, ethanol and acetone have been used as cosolvents.

Cosolvent addition into a SCFE system can be accomplished in several ways. The most common method reported in the literature (Wong and Johnston, 1986) seems to be the introduction of a cosolvent/solvent mixture into a SCFE system through a reservoir containing known amounts of solvent and liquid cosolvent. Although gas tanks containing desired levels of cosolvent are commercially available, their high cost limits the feasibility of this approach. Another disadvantage of premixed gas tanks is the variation in mixture composition with time due to decreasing reservoir pressure throughout the extraction process, which changes cosolvent solubility in SCF. Treatment of the sample to be extracted with a known amount of cosolvent and equilibration of the mixture overnight at 4°C, prior to the extraction process, is another approach in cosolvent addition. However, this method also has disadvantages. Maintaining constant cosolvent concentration level in the system is not possible, since cosolvent is carried out with the supercritical solvent and its concentration decreases with time during the extraction process (Temelli, 1992).

Schmitt and Reid (1986) maintained a constant SCF solvent flow rate throughout the extraction period by adjusting flash valves on the SCF solvent line as required and monitored the flow rate by a rotameter. Cosolvent was injected into the solvent gas at a constant volumetric flow rate through a high-pressure chromatography pump to achieve the desired mixture composition. Schmitt and Reid (1986) reported that experimental data reflected the difficulty in achieving a constant mole percentage of cosolvent at different system pressures since it was difficult to maintain a constant flow rate of gas through the heated expansion valves (sampling and flash valves).

Recently, an advanced solvent-cosolvent mixing system has been developed by Dionex Inc. (Salt Lake City, UT) for their analytical scale instrumentation. This system consists of a computer controlled high pressure pump capable of measuring solvent density at the cosolvent injection point. Thus, the corresponding amount of cosolvent to achieve a desired mixture concentration is injected into SCF automatically.

Non-toxic ethanol is the preferred cosolvent for food applications. Staby (1993) reviewed the literature on the phase equilibria of CO_2 + alcohol systems. SC-CO_2 extraction of palm oil (Brunner and Peter, 1982b), gluten and rice bran (Hammam and Sivik, 1991), lipids from fish muscle (Hardardottir and Kinsella, 1988), and extraction and fractionation of egg yolk (Bulley et al., 1992) are examples where increased solubility of lipids in the presence of ethanol in the supercritical phase was reported. Ethanol also enhances the solubility of polar compounds, such as PL, in SC-CO_2 (Hammam et al., 1987; Hardardottir and Kinsella, 1988; Hammam and Sivik, 1991; Bulley et al., 1992; Temelli, 1992).

Water content of the samples can be an important factor influencing the SCF extractability of the desired compounds. For example, although caffeine is soluble in SC-CO_2, it can be removed from whole raw beans only when the beans are moist (Zosel, 1981). However, water content of the feed material might have a negative effect on the SCF extraction yield. King et al. (1989) indicated that yield of fat extraction with SC-CO_2 decreased with increasing moisture content of the beef samples. In the presence of water in fat (butter), cholesterol solubility in SC-CO_2 increased up to 20.0 MPa, then decreased with a further pressure increase (Q.P. Corp., 1987). Moisture content at a level of 10% in oilseeds does not affect the solubility of oil in SC-CO_2 significantly (Snyder et al., 1984). However, water does play some part by increasing the permeability of the cell membrane through swelling effects, hence improving the accessibility of the oil. A portion of the water present in the samples is co-extracted with the oil by SC-CO_2 (Von Eggers and Stein, 1984; Snyder et al., 1984; Chao et al., 1991). During essential oil extraction, polar component solubilities in SC-CO_2 were improved at ~20% (w/w) sample moisture content (Stahl and Gerard, 1985; Gopalakrishnan and Narayanan, 1991).

1.3.2.2.4. Fish and proteins

The first report on the application of SCFE to marine lipids was the Solexol process for vitamin A concentration from fish oils in 1946 (McHugh and Krukonis, 1994). Later, menhaden oil (Passino, 1949; Dickinson and Meyers, 1952), sardine and cod liver oils (Passino, 1949) were fractionated using propane. Ethane was also used to extract and fractionate fish liver oil (Zosel, 1978). Concentration of ω-3 fatty acids in fish oil using SC-CO_2 have been studied extensively (Eisenbach, 1984; Krukonis, 1988; Rizvi et al., 1988; Nilsson et al., 1988, 1989; Higashidate et al., 1990). Supercritical CO_2 has also been used to extract oil directly from fish muscle of Antarctic krill (Yamaguchi et al., 1986), rainbow trout (Hardardottir and Kinsella, 1988) and Atlantic mackerel (Temelli et al., 1995).

Effect of SC-CO_2 on protein and enzymes varies with pressure, temperature and feed composition. Taniguchi et al. (1987a) studied the effect of SC-CO_2 treatment (20.3 MPa, 35°C and 1h) on the activities of several enzymes. All the enzymes studied retained over 90% of their activity. However, it was also shown that peroxidase (Christianson et al., 1984), pectinesterase (Balaban et al., 1991) and polyphenol oxidase (Chen et al., 1992) were inactivated following SCFE. Ribonuclease (Weder, 1980) and lysozyme (Weder, 1984) were slightly denatured by SC-CO_2. Taniguchi et al. (1987b) were able to extract oil from mustard seeds with SC-CO_2 without lowering the myrosinase activity and sinigrin content of the samples.

1.4. OBJECTIVES OF THE THESIS

Even though extraction of oil from various oilseeds has been studied extensively, optimization of all processing parameters needs further investigation. Development of an industrial scale operation is not possible, until we have an understanding of the interactions between processing parameters and important chemical constituents in the feed material. It has been established that PL are not extracted with SC-CO_2 during oil extraction and that they are left behind in the meal. Recovery of these valuable components with the addition of a polar cosolvent was not reported when this study was initiated. As well, our knowledge on the effect of moisture on lipid recovery, extract composition, residual proteins and their enzymatic activity is limited. For this purpose, canola and Atlantic mackerel were chosen as feed materials to represent low- and high moisture biological resources, respectively, that are important for the Canadian economy. Thus, this study examines various aspects of processing parameters such as temperature, pressure, use of ethanol as an entrainer, moisture content of feed materials, feed heat pretreatments on the SC-CO_2 extraction of edible oils with emphasis on the lipid composition of extracts and residual proteins. The specific objectives of this research were:

1) to investigate the use of SC-CO_2/EtOH mixtures to extract PL from flaked canola seeds, canola meal and the acetone insolubles (AI) fraction obtained from crude canola lecithin, and to examine the effects of processing parameters such as temperature, pressure and ethanol concentration on PL recovery and the PC content of the extracts (chapter 2);

2) to investigate the effect of moisture content and heat pre-treatment of canola flakes and extraction conditions such as temperature and pressure on the lipid composition of SC-CO_2 extracts (chapter 3);

3) to determine the effect of temperature, pressure and feed moisture content on the endogenous canola enzyme myrosinase (thioglucoside glycohydrolase, E.C.3.2.3.1)

activity and the extent of glucosinolate hydrolysis during the SC-CO_2 treatment of canola seed and flakes (chapter 4); and

4) to study the effect of moisture content of Atlantic mackerel on oil extractability with SC-CO_2 and also to examine the effect of fish moisture content on the composition of lipid extract and residual proteins (chapter 5).

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TABLE 1.1. Lipid composition of various oilseeds[1].

Oilseed	Total lipid (%, w/w)	Total phospholipid (% of total lipids, w/w)
Canola[2]	40-44	1.5-3.6
Soybean	18-20	1.1-3.2
Cottonseed	18-20	0.7-0.9
Peanut	45-50	0.3-0.4

[1] Adapted from Allen et al. (1979).
[2] Adapted from Eskin (1987).

TABLE 1.2. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) ratios of various seeds[1].

	PC (%)	PE (%)
Canola[2]	84.4	15.6
Sunflower	73.1	26.9
Soybean	63.1	36.9
Alfalfa	82.4	17.6

[1] Adapted from Weihrauch and Son (1983).

[2] Adapted from Eskin (1987).

TABLE 1.3. World trade for 10 major fats and oils (x1000 metric tons)[1].

Product	Production[2]	Consumption[2]	Export
Rapeseed /canola	9409	9517	1941
Soybean	16947	16780	4328
Sunflower	8080	8090	2477
Olive	2175	1955	713
Corn	1566	1525	494
Palm	12024	12199	8574
Coconut	2894	2898	1549
Groundnut	3961	3950	358
Cottonseed	4265	4340	275
Fish	1047	1143	609

[1] Adapted from Anon. (1993a).

[2] Balance between production and consumption was supplied from stocks.

TABLE 1.4. Fatty acid composition of selected oils[1].

Fatty acid	Canola[2]	Soybean	Rapeseed	Corn	Atlantic mackerel[3]
C16:0	3.7	12.0	4.0	12.0	15.9
C16:1	-	0.5	-	-	7.4
C18:0	1.7	3.6	1.6	2.0	3.6
C18:1 (ω-9+ω-7)	60.9	23.7	24.0	26.0	22.7
C18:2	19.6	51.4	13.6	59.0	-
C18:3 (ω-6+ω-3)	12.4	8.8	12.5	1.0	2.4
C20:0	0.5	-	-	-	11.2
C20:1	1.4	-	7.0	-	1.9
C20:5 (ω-3)	-	-	-	-	20.3
C22:1	0.9	-	37.0	-	-
C22:5	-	-	-	-	1.3
C22:6 (ω-3)	-	-	-	-	8.6

[1] Adapted from Allen et al. (1979)

[2] Adapted from Temelli (1992)

[3] Adapted from Temelli et al. (1995)

TABLE 1.5. Critical temperature and pressure of common fluids[1].

Fluid	Critical Temperature T_c (°C)	Critical Pressure P_c (MPa)
CO_2	31.0	7.3
Chlorotrifluoromethane	28.9	3.8
Ethylene	9.3	5.1
Ethane	32.2	4.8
Propane	96.8	4.2
Propylene	91.9	4.5
Cyclohexane	280.4	4.0
Isopropanol	235.2	4.7
Benzene	288.9	4.9
Toluene	320.8	4.2
p-Xylene	345.0	3.4
Trichlorofluromethane	198.0	4.3
Ammonia	132.5	11.3
Water	374.1	21.8

[1] Adapted from Weast (1977-78).

TABLE 1.6. Fatty acid composition of hexane and $SC\text{-}CO_2$ extracted oils[1].

Fatty acid	Canola[2]		Soybean[3]		Atlantic mackerel[4]	
	Hexane extract	$SC\text{-}CO_2$ extract[5]	Hexane extract	$SC\text{-}CO_2$ extract[6]	Hexane extract	$SC\text{-}CO_2$ extract[7]
C16:0	3.7	3.7	10.8	11.0	15.9	16.7
C16:1	-	-	-	-	7.4	8.8
C18:0	1.7	1.9	4.2	3.5	3.6	3.2
C18:1 (ω-9+ω-7)	60.9	61.8	27.0	27.5	22.7	38.7
C18:2	19.6	17.6	51.4	52.0	-	-
C18:3 (ω-6+ω-3)	12.4	13.6	6.6	6.0	2.4	3.0
C20:0	0.5	-	-	-	11.2	7.8
C20:1	1.4	1.5	-	-	1.9	1.2
C20:5 (ω-3)	-	-	-	-	20.3	18.9
C22:1	0.9	-	-	-	-	-
C22:5	-	-	-	-	1.3	1.4
C22:6 (ω-3)	-	-	-	-	8.6	9.0

[1] Gas chromatography area %
[2] Adapted from Temelli (1992)
[3] Adapted from Friedrich and List (1982)
[4] Adapted from Temelli et al. (1995)
[5] Extracted at 62.1MPa and 70°C
[6] Extracted at 34.5 MPa and 50°C
[7] Extracted at 34.5 MPa and 35°C

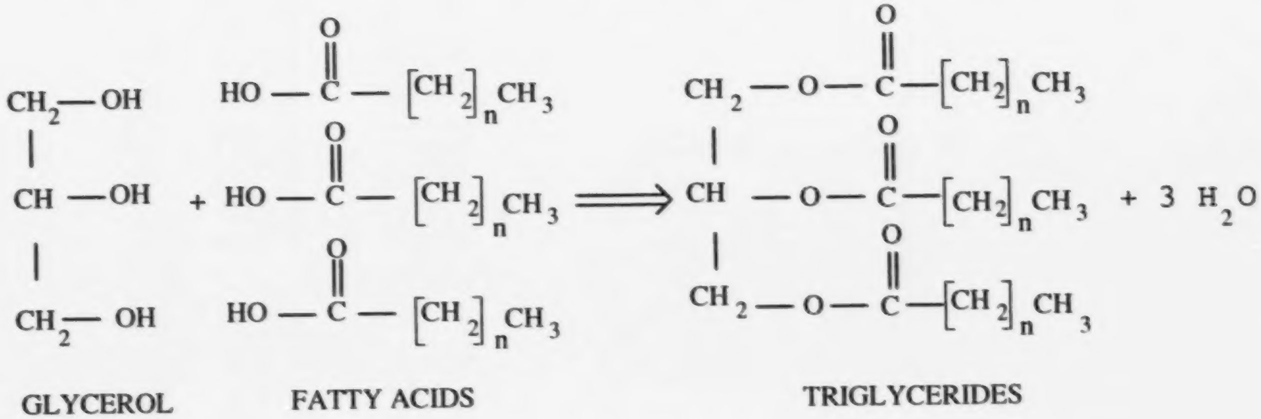
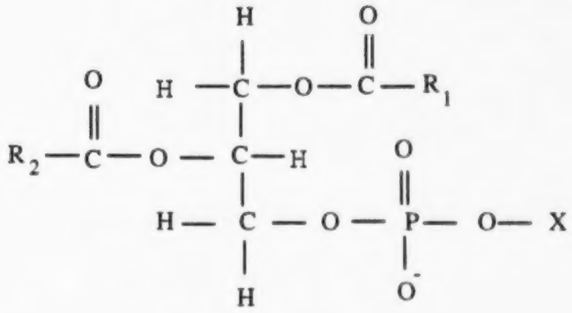
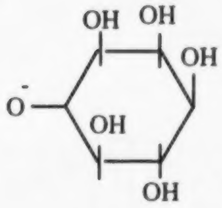
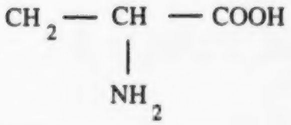


FIGURE 1.1. Chemical structure of triglycerides.



Phosphatidyl Unit	X
Phosphatidic acid	H
Choline	$CH_2—CH_2—N^+[CH_3]_3$
Ethanolamine	$CH_2—CH_2—N^+H_3$
Inositol	
Serine	 $\underset{\displaystyle NH_2}{CH_2—CH—COOH}$

R_1 and R_2 are fatty acid groups

FIGURE 1.2. Chemical structure of phospholipids.

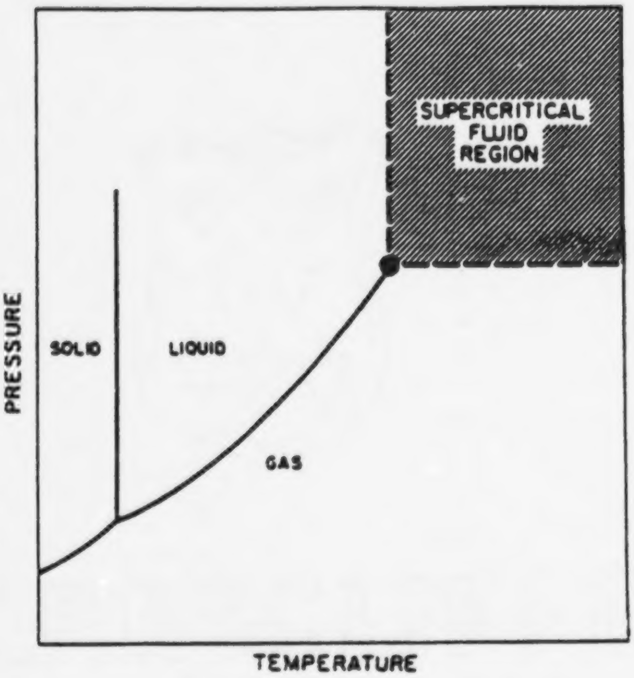


FIGURE 1.3. Phase diagram for a pure substance.

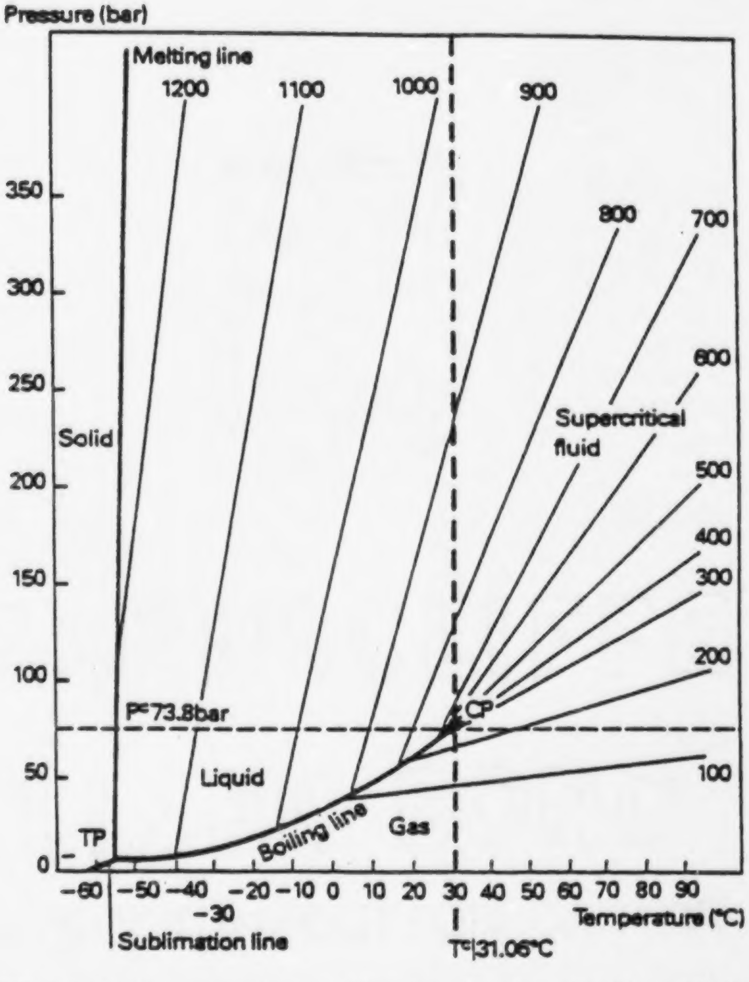


FIGURE 1.4. Pressure-temperature diagram of carbon dioxide with the density as a third dimension. Densities are given from 100 to 1200 g/L (Brogle, 1982).

2. EXTRACTION OF PHOSPHOLIPIDS FROM CANOLA WITH SUPERCRITICAL CARBON DIOXIDE AND ETHANOL[1]

2.1. INTRODUCTION

The term lecithin refers to a by-product of the edible oil industry, consisting of a mixture of phospholipids (PL) mixed with vegetable oil. Lecithins produced from egg yolk and soybeans are commercially available. Although canola has slightly higher phospholipid concentration than soybeans (1.5-3.6 wt % vs 1.1-3.2 wt %), it is not used for commercial lecithin production. Lecithin is an important natural emulsifier which is used in the food, feed and pharmaceutical industries. Crude lecithin has both weak water-in-oil (W/O) and oil-in-water (O/W) emulsifying properties due to the constituent PL. To improve its properties for specific product applications, crude lecithin is modified, refined and/or fractionated.

Demand for lecithin with a high phosphatidylcholine (PC) content has increased in the cosmetic, pharmaceutical, food and other industries (Juneja et al., 1989). High PC lecithin has better O/W emulsifying properties and functions over a wider pH range than crude lecithin. PC is also reported to have beneficial therapeutic effects such as lowering cholesterol levels and treatment of neurological disorders (Hanin, 1979). Industrial scale modification of crude lecithin is performed by physical, chemical and enzymatic methods (Van Nieuwenhuyzen, 1976). Commercially deoiled lecithin is obtained by treating crude lecithin with acetone. Acetone deoiling of crude canola lecithin was reported by Temelli and Dunford (1995). The disadvantages of this process are the use of high temperatures for drying the product, as well as incomplete solvent removal.

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Supercritical fluid extraction (SCFE) of crude lecithin is a relatively new process. Crude lecithin, when extracted with supercritical carbon dioxide (SC-CO_2), produces a deoiled lecithin without any residual solvent. Heigel and Hueschens (1983) have patented a SC-CO_2 extraction process for the production of deoiled lecithin from crude soybean lecithin which contained 30% (w/w %) oil. When crude lecithin was extracted with CO_2 at 60°C and 40 MPa for 4 hours, a solid lecithin was obtained having a light yellow color. It was indicated that this product could be used directly for medicinal purposes and its physical properties such as aroma, color etc., were better than those of the products obtained by conventional methods. However, when commercial liquid lecithin was extracted with SC-CO_2 at 30-40 MPa and 32-60°C by Castera (1994), a product was obtained that was darker in color, with less soya odor than their product of acetone insolubilization. Alkio et al. (1991) obtained a crystalline or semiplastic product from crude oat lecithin after extracting the oat oil with SC-CO_2. This product had acceptable flow properties and appearance eliminating the need for any additives. The PL concentration of oat lecithin, obtained by SC-CO_2 extraction (30-35%, w/w %), was lower than that of the soybean lecithin (50%, w/w %) obtained by the same technique. However, oat lecithin exhibited better antioxidant properties than soybean lecithin.

A continuous near-critical fluid extraction process using CO_2 has been developed to remove oil from crude soybean lecithin (Peter et al., 1985), yielding a solvent free powder, yellow-white in color. In this and another study (Von Stahl and Quirin, 1985), design and the operation of a continuous SCFE pilot plant system for deoiled lecithin have been described. Improvement of contact between the solvent and viscous lecithin under high pressure by adding crude lecithin on an inert carrier material, or the addition of organic solvents were advocated. The use of stirred extractors, or development of a high pressure jet injection system for introducing the crude lecithin into the solvent stream, were other options discussed (Von Stahl and Quirin, 1985). Eggers and Wagner (1993) developed a semi-industrial scale apparatus to bring viscous lecithin into contact with SC-CO_2 in a special spraying device. While the above mentioned studies focused on oil removal from crude lecithin, List et al. (1993) developed a counter-current process to degum crude soybean oil. In this study, the phosphorus content of crude oil was reduced from 620 ppm to <5 ppm with SC-CO_2 at 55 MPa and 70°C.

The supercritical solvent of choice for food applications, carbon dioxide, exhibits a low solubility for the relatively polar PL. It has been reported that SC-CO_2 does not extract PL from crushed oil seeds (Friedrich and Pryde, 1984; Fattori et al., 1987). Soybean and canola oil extracted with SC-CO_2 contain less than 100 ppm PL while a hexane extracted oil contains approximately 1% (w/w %) PL (Friedrich and Pryde, 1984;

Fattori et al., 1987). Thus, when SC-CO_2 is used for oil extraction, valuable PL are left behind in the meal. However, the addition of a polar cosolvent would enhance the selectivity of the supercritical solvent towards the PL. Temelli (1992) has shown the presence of PL in the extracts obtained from canola flakes and press cake with SC-CO_2/EtOH mixture following SC-CO_2 extraction of oil, using thin layer chromatography. As well, Bulley et al. (1992) demonstrated that SCFE of PL from freeze-dried egg yolk using methanol or ethanol as a cosolvent (at 3-5%, w/w %) in SC-CO_2, yielded extracts containing 6.8 to 17% (w/w %) PL. More recently, there has been simultaneous efforts focusing on the recovery of PL from various oilseeds such as: canola (Dunford and Temelli, 1994), soybean (Montanari et al., 1994) and cottonseed (Sivik et al., 1994). Montanari et al. (1994) used a consecutive two step SCFE process to deoil soybean flakes and to isolate PL-enriched fractions. These studies demonstrated that the addition of ethanol as a cosolvent into SC-CO_2 allows selective recovery of polar PL from oilseeds but optimization of processing parameters and fractionation of PL mixtures need further investigation.

The objectives of this study were: a) to investigate the use of SC-CO_2/EtOH mixtures to extract PL from flaked canola seeds, canola meal and the acetone insolubles (AI) fraction obtained from crude canola lecithin and b) to examine the effects of processing parameters such as temperature, pressure and ethanol concentration on PL recovery and the PC content of the extracts.

2.2. MATERIALS AND METHODS

Crude canola (*Brassica napus* and *Brassica campestris*) lecithin, flaked canola seeds and canola meal were obtained from Canamera Inc., Fort Saskatchewan, AB and were kept below -20°C until used. Headspace of the crude canola lecithin containers was flushed with nitrogen before storage.

Oil content of the flaked canola seeds was determined according to the AOCS Official method Ac 3-44 (AOCS, 1990). Flaked canola seeds and canola meal were extracted with chloroform/methanol (2:1, v/v) mixture according to the method of Sosulski et al. (1981) to determine phospholipid content.

AI were obtained by treating 25 g of crude canola lecithin with acetone (99.5% purity, Omni Solv., BDH Inc., Toronto, ON) at room temperature as reported previously (Temelli and Dunford, 1995). Oil was removed from crude lecithin using three consecutive extraction steps, consisting of the addition of 150, 100 and 100 mL acetone, followed by 1 h of stirring for each stepwise addition. AI were filtered after each step, pooled and dried in a vacuum oven at 40°C for 24 hours. All dried samples were stored at <0°C.

Supercritical fluid extraction system and its modification

A laboratory scale SCFE unit designed and manufactured by Newport Scientific Inc. (Jessup, MD) was used for this study. The extraction vessel (300 mL, 300SS) was heated by two silicon-fibreglass electrical resistance type heating tapes (100 W each), with a burnout temperature of 232°C and temperature was controlled by a thermostat (±

2°C) via a type J thermocouple immersed at the centre of the extractor. A stainless steel basket which fits into the extraction vessel, was fabricated for ease of loading and unloading the sample. The ends of the basket were mounted with two 10 μ metallic filters (Mott Metallurgical Co., Farmington, CA) to avoid any sample carry over. Carbon dioxide (99.9% (w/w %) purity, bone dry, Medigas Alberta Ltd., Edmonton, AB) was compressed to the desired pressure by a diaphragm compressor with a maximum rating of 69.0 MPa. Pressure at the compressor inlet and outlet (extraction vessel) was measured through two bourden tube gauges. The extraction pressure was controlled by a back pressure regulator (Tescom Co., Elk River, MN). A rupture disc was provided as pressure overshoot protection. The system was equipped with a needle valve for depressurization of SC-CO_2 and sample collection. The flow rate of the gas passing through the extractor was controlled by periodic manual adjustment of this expansion valve. The extract was collected in glass tubes attached after the depressurization valve which were held in a refrigerated circulating bath (Lauda, Model RMT-6, Brinkmann, Rexdale, ON) at -15°C. Depressurized CO_2 was passed through a rotameter and the volume of carbon dioxide used was recorded by a dry gas meter (American Meter Co., Horsham, PA) at ambient temperature (~20°C) and pressure (0.1 MPa) before venting to the atmosphere. Average mass flow rate of CO2 was reported. A bed of silica gel was placed between the sample collection tubes and the rotameter to prevent contamination of the rotameter and flow totalizer.

The original SCFE unit presently described, which did not have cosolvent addition capability, was modified for cosolvent introduction into the supercritical solvent. Among the different approaches discussed in Chapter 1, continuous injection of cosolvent into CO_2 using a high pressure liquid pump was the method adopted. Thus, ethanol (99.9% purity, Commercial Alcohol Ltd., Montreal, QC) was pumped into the system by a high pressure piston pump (Model 305, Gilson Inc., Middleton, WI). Addition of ethanol into CO_2, before or after CO_2 is pressurized were the options examined. A series of test runs were carried out with ethanol injection before and after the compressor. Ethanol introduction into the CO_2 line before the compressor had several disadvantages: a) longer retention time of ethanol in the system before sample-cosolvent contact, b) cosolvent accumulation in the system due to recycling of solvent by the back pressure regulator, c) contact of ethanol with compressor and back pressure regulator parts, and d) the need to pump liquid CO_2 instead of gas to avoid the presence of two phases in the compressor head which requires cooling of the CO_2 line. It was concluded that addition of ethanol into pressurized CO_2 before it enters the extraction cell provided better control of solvent mixture composition. A flow diagram for the modified SCFE unit is shown in Figure 2.1.

Although the maximum pressure obtainable on the SCFE unit was 69.0 MPa, a maximum pressure of only 55.2 MPa was used for the experiments involving ethanol due to the lower pressure limit of the cosolvent addition pump. Ethanol was pumped into the system at a constant predetermined volumetric flow rate. The mole percent of ethanol continuously pumped into the SC-CO_2 during the extraction period was reported as the average based on the total amount of CO_2 passed through the system for each run due to slight fluctuations in the CO_2 flow rate as ethanol separated in the depressurization valve. The quantity of the various extracts was determined gravimetrically. For the cosolvent based runs, ethanol was removed from the final extract by rotary evaporation under vacuum at a temperature of 45±3°C. Ethanol evaporation was continued until the oil extract maintained constant weight in three successive measurements.

Canola flakes

The extractor cell was loaded with 45 g flaked canola seeds for each experiment. SCFE experiments were performed using temperature and pressure ranges of 45-70°C and 41.1-62.1 MPa, respectively. Two sets of experiments were carried out with canola flakes. Full-fat canola flakes were used for the first set of experiments. SC-CO_2 extractions of full-fat canola flakes were performed, with and without the addition of ethanol (8 mole %), as cosolvent. The second set of experiments were carried out with canola flakes having a reduced oil content. To affect this oil reduction, full-fat canola flakes were extracted with SC-CO_2 at 62.1 MPa and 70°C, until the oil content was reduced to <15% (w/w %), prior to the addition of ethanol into the system at 5 or 10% levels. These extraction conditions were determined to give maximum oil solubility in SC-CO_2 within the temperature and pressure ranges studied using the same unit (Temelli, 1992). All canola flake extractions were carried out for 3 h.

Canola meal

Sixty-five grams of canola meal were used for each experiment. Initially, experiments were carried out using SC-CO_2/EtOH (5-9 mole %) mixture at 55.2 MPa and 70°C. A second set of experiments was performed by soaking the canola meal. For the solvent soaking experiments, SC-CO_2 and ethanol were added to the system in two stages. During a pre-extraction period, CO_2 was pumped into the system until the designated extraction pressure and temperature were reached. During this initial pressurization, 50 mL of ethanol was pumped into the extraction cell over a 2 min period. The compressor was then allowed to run for 90 min to improve the mixing of CO_2 and ethanol. This period of the extraction was referred to as the "soaking" step since the depressurization valve was closed.

The second stage of the extraction was started after opening the depressurization valve, followed by CO_2 pumping for 1.5 h at a flow rate of 6.7±0.4 g/min at 55.2 MPa and 45-70°C. Throughout this extraction stage, ethanol was mixed into the CO_2 by the Gilson pump at a constant flow rate to achieve 7.5 mole % in SC-CO_2.

Canola acetone insolubles

SCFE of AI were carried out within the temperature and pressure ranges of 45-70°C and 20.7-55.2 MPa, respectively. Twenty grams of canola AI were used in each experiment. Experiments with or without soaking were performed similar to the above canola meal experiments using the following parameters: The quantity of ethanol used for soaking was varied from 0-60 mL for a soaking period of 60 min, keeping the CO_2 flow rate at 2.0±0.3 g/min.

Phospholipid analysis

Total PL content of the samples was determined by perchloric acid digestion followed by spectrophotometric quantitation of phosphorus according to the Bartlett method (1959) as modified by Marinetti (1962). A conversion factor of 25.6 was used to convert total phosphorus to PL amounts. Calculation of the conversion factor was based on the PL composition of canola AI reported by Vaisey-Genser and Eskin (1987). Molecular weights of individual canola phospholipids were taken from Fattori et al. (1987).

Separation of PL components and quantification based on peak areas were done by an Iatroscan TH-10 Mark II analyzer equipped with precoated silica gel chromarods SII (T.M.A. Scientific Supply, Mississauga, ON) according to the method of Ratnayake and Ackman (1985). The chromarods were developed in two steps wherein acetone was used first to separate the non-polar compounds followed by a polar solvent system of chloroform:methanol:water (65:35:4 v/v/v). Chromarods were scanned under the following conditions: hydrogen pressure 73.5 kPa, air flow rate 2 L/min. Peak areas were integrated with the Hewlett-Packard Series 3356 laboratory automation system (Hewlett-Packard, Wilmington, DE). Phospholipid standards (Sigma Chemical Co., St. Louis, MO); PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) were used for peak identification. PC concentration in each extract was reported as the area percentage of the total Iatroscan peak areas for all PL fractions in the sample. Lipid standards and samples were kept at -80°C until analysis.

Statistical analysis

All extraction runs and analysis of each extract were carried out in duplicate in randomized order and means were reported. Analysis of variance of the results was performed using General Linear Model procedure of SAS Statistical Software, Version 6 (SAS, 1989). Multiple comparison of the means were carried out by LSD (Least Significant Difference) test at $\alpha = 0.05$ level.

2.3. RESULTS AND DISCUSSION

Oil, PL and PC content of the starting materials are given in Table 2.1.

Canola flakes

When full-fat canola flakes were extracted with SC-CO_2 at 55.2-62.1 MPa and 45-70°C, no phosphorus was detected in the extracts. These results were in agreement with previous findings (Friedrich and Pryde, 1984; Fattori et al., 1987), confirming the higher selectivity of SC-CO_2 for nonpolar triglycerides (TG) relative to polar phospholipids in the presence of large amounts of TG.

Addition of 8% ethanol into the SC-CO_2 during the extraction of full-fat canola flakes resulted in a PL concentration of <200 ppm in the oil extracted at 55.2 MPa and 70°C. This indicated that the selectivity of SC-CO_2 for TG was not affected by the presence of ethanol at 8% level when full-fat canola flakes were extracted.

Friedrich et al. (1982) reported that the phosphorus concentration of SC-CO_2 extracted soybean oil increased in the final oil fractions. While earlier fractions (first 10-20%) contained less than 20-25 ppm phosphorus, the last fraction (last 10%) had ~95 ppm phosphorus. Similar results have been obtained on freeze-dried egg yolk (Bulley et al., 1992). When canola flakes of reduced oil content were extracted with SC-CO_2 in the presence of ethanol, PL were detected in the extracts (Table 2.2). When the oil content of canola flakes was reduced from 23.0% to 12.0%, there was a significant drop ($p<0.05$) in the amount of SC-CO_2 extract. CO_2 loading at 43.0% oil was the highest, 9.7 mg oil/g CO_2, due to the lower CO_2 flow rate compared to the other extraction runs (Table 2.2) which resulted in longer contact time between sample and SC-CO_2. The highest canola oil saturation level in SC-CO_2 was reported as 43.3 mg oil/g CO_2 at 62.0 MPa, 70°C and flow rate of 1.2 g CO_2/min for EtOH-free SC-CO_2 (Temelli, 1992). However, oil saturation concentrations in SC-CO_2/EtOH mixtures are not available at extraction conditions comparable to our study. Even though PL content of these extracts showed an increasing trend, differences were not statistically significant ($p>0.05$). Further deoiling of the canola flakes beyond ~14% (w/w %) was not attempted since attainable extraction rates in this region were very low with the available SCFE unit. However, further deoiling of the canola flakes with EtOH-free SC-CO_2 should increase PL extraction efficiency with SC-CO_2/EtOH in the second step. Experiments carried out at 55.2 MPa, 70°C and two levels of ethanol addition showed that both the amount and the PL concentration of the extracts increased significantly ($p<0.05$) with increasing ethanol percentage in SC-CO_2 (Table 2.2). Doubling the amount of ethanol added, from 5 to 10%, resulted in a 10-fold increase in the PL concentration of the extracts. The amount of extracts collected at different temperature and pressures varied between 1.5-2.7 g (Table 2.3). There was a significant ($p<0.05$) increase in the amount of extract with temperature at 55.2 MPa. PL concentrations of the extracts were between 4.0-5.4% (Table 2.3) and they were similar ($P>0.05$).There appears to be an anomaly in PL content of extracts obtained at 55.2 MPa and 55°C.

Canola meal

Although the composition of canola lecithin (Vaisey-Genser and Eskin, 1987) is similar to that of the soybean lecithin (Van Nieuwenhuyzen, 1976), canola lecithin has not been previously utilized and is added back to the meal which is used as animal feed. During commercial processing of the canola flakes, the flake oil content is reduced to 1-2% by hexane extraction. However, addition of crude canola lecithin back into the residue increased the total lipid content of the meal up to 8% (Table 2.1). To study SC-CO_2/EtOH extraction of PL, hexane extracted canola flakes were used as a solid support for crude lecithin. Such a solid support would provide better contact between the SC solvent and the lecithin since solvent-feed contact (due to the gummy consistency of crude lecithin) had been a major problem during the SCFE of crude lecithin. When canola meal was extracted with SC-CO_2/EtOH mixture, both amount and phospholipid concentration of the extracts increased significantly ($p<0.05$) with increasing ethanol percentage in the SC-CO_2 (Table 2.4).

In an effort to increase the amount of extract and its PL concentration, canola meal samples were soaked with ethanol under 55.2 MPa prior to extraction. In this case, both PL concentration and the amount of extract increased with increasing extraction temperature, even though it did not reach significant levels ($p>0.05$) (Table 2.5). When the canola meal was soaked for 90 min at 55.2 MPa and 70°C and then extracted with SC-CO_2/7.5% EtOH, it was possible to obtain extracts with a PL concentration of ~38.6% (w/w % of extract) (Table 2.5). Under these conditions, 1.9 g of extract was collected. The effect of pressure on the soaking treatment was examined by treating canola meal with ethanol at atmospheric pressure for 6 h prior to SC-CO_2 extraction. In the case of atmospheric soaking, the amount of extract was slightly higher, 2.4 g, but PL concentration of the extract was lower, 34%, compared to that for soaking under pressure. Longer soaking time allowed at atmospheric pressure resulted in solubilization and extraction of a larger amount of lipids and PL which seem to be diluted.

Canola acetone insolubles

Extraction of canola AI with SC-CO_2 in the presence of ethanol was investigated in an effort to fractionate the PL components. The effect of temperature and pressure on the AI extracts obtained without prior soaking were examined at 55.2 MPa and 70°C, respectively, because experiments carried out with meal at these conditions gave the highest extract amount and PL concentration in the extracts. The highest PL concentration in the extracts of ~42%, was reached at 55.2 MPa and 45°C (Table 2.6).

PL concentration of the extract obtained at 70°C and 55.2 MPa was significantly ($p<0.05$) lower than that of the extracts obtained at lower pressures and at the same temperature (Table 2.6). The major problem with AI extractions was the formation of a cake in the extractor. Therefore, this caking problem has to be solved before any further investigation of optimum processing conditions can be adressed.

AI samples were soaked with ethanol at 55.2 MPa and 70°C and then extracted with SC-CO_2/13.0 or 6.5% EtOH, and results are presented in Table 2.7. Extraction conditions for the soaking experiments carried out with AI were based on the extraction conditions (70°C and 55.2 MPa) at which highest extract amount and PL concentration were obtained for the previous experiments carried out with canola flakes and meal. Even though the amount of extract showed a slight increase, the effect of ethanol percentage in SC-CO_2 was not significant ($p>0.05$). The effect of the amount of ethanol used for soaking on the PL concentration of the extracts was significant ($p<0.05$) (Table 2.7) and PL concentration increased with ethanol level. A maximum PL concentration of 50% was obtained with a SC-CO_2/6.5% EtOH mixture at 55.2 MPa and 70°C, with 60 mL of ethanol used for soaking. Higher PL content of extracts obtained from canola meal (Table 2.5) with soaking compared to AI also support the fact that solvent-feed contact was hindered by cake formation in the extractor. Use of a stirred extractor should improve solvent-feed contact.

PC content of the samples increased with increasing PL concentration in the extracts. The canola flake extracts with 5.4% PL (Table 2.2) obtained at 55.2 MPa and

70°C had only 4.2% PC. PC concentration as high as 72.7% was reached in the meal extracts with 39% PL obtained at 55.2 MPa and 70°C with 90 min of ethanol soaking (Table 2.5). On the other hand, extracts of canola meal soaked at atmospheric pressure obtained at the same extraction conditions had only 19.0% PC. Thus, higher diffusion rates and solvent power of CO_2 under supercritical conditions must be contributing to the extraction of PC out of the cell matrix during the soaking period. With canola AI, the highest PC concentrations were reached in the extracts with 50% PL content which were obtained with 60 mL ethanol soaking and SC-CO_2/6.5% EtOH extraction. In these samples, 90% of the PL extracted were PC (Table 2.7). The extracts which had the lowest PL concentration also had the lowest PC concentration. Even though PC content of the extracts increased with the level of ethanol addition (Table 2.7), differences were not statistically significant ($p>0.05$). These results can be explained in relation to the higher solubility of PC in ethanol compared to other PL components.

Sample material balance and percentage recovery calculations are given in Figure 2.2. Maximum PL recovery of 30.4% was reached with meal samples soaked with 50 mL ethanol followed by SC-CO_2/7.5% EtOH extraction at 55.2 MPa and 70°C for 3 h (Fig. 2.2b). For the same samples PC recovery (54.5%) was higher than the PL recovery indicating higher selectivity for PC. The highest attainable PL recovery for canola flakes was 20.8% (Fig. 2.2a). The maximum PL recovery from soybean flakes reported by Montanari et al. (1994) was 6.7% with SC-CO_2/10.2% EtOH at 68.2 MPa and 80°C. PL and PC recoveries were very low for AI extracts obtained with or without soaking due to caking of AI in the extractor cell in both cases (Fig. 2.2c).

2.4. CONCLUSIONS

This study demonstrated that PL can be recovered in a second step with SC-CO_2/EtOH mixture after oil extraction is affected with EtOH-free SC-CO_2. The oil content of the seeds must be lowered as much as possible in the first step to increase the efficiency of PL extraction as well as to minimize oil in the PL extracts. Soaking of feed material with ethanol was a new approach in an effort to improve PL recovery and fractionation, inspired by the fact that coffee beans are saturated with water to release caffeine prior to caffeine extraction with SC-CO_2 saturated with water in a commercial operation (McHugh and Krukonis, 1994). Soaking of meal with ethanol improved PL recovery to 30.4% compared to 20.8% from flakes without soaking. The effect of the amount of EtOH used for soaking of AI on the PL concentration of the extracts was significant ($p<0.05$). PL concentration and amount of SC-CO_2 extracts obtained from canola meal and flakes increased significantly ($p<0.05$) with increasing amount of ethanol in the SC-CO_2.

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TABLE 2.1. Oil, phospholipid (PL) and phosphotidylcholine (PC) concentration of the feed material for extraction.

	Oil content (w/w %)	PL content (w/w %)	PC content (area %)[1]
Flakes	43.0[2]	3.7	n.d.[3]
Meal	8.0[4]	46.0	41.5
Acetone insolubles	n.d.[3]	72.0	41.6

[1] Based on the total Iatroscan peak area of all PL fractions.
[2] Determined according to Official Method of AOCS (1990).
[3] not determined.
[4] Total lipids determined according to Sosulski et al. (1981).

TABLE 2.2. Effect of the triglyceride content of canola flakes and ethanol concentration on the SC-CO_2/EtOH extracts at 55.2 MPa.

Temperature (°C)	70^1	70^1	55^1	55^1	70^2
Oil content of flakes (w/w %)	14.0	14.0	12.0	23.0	43.0
Ethanol % in SC-CO_2 (mole %)	5.0	10.1	10.3	10.1	8.3
Amount of extract (g)	1.0^a	2.7^b	2.3^e	6.4^f	3.5
Phospholipid content of extracts (w/w %)	0.5^c	5.4^d	1.3^g	0.7^g	n.d.[3]

[1] CO_2 flow rate = 5.4 g/min.
[2] CO_2 flow rate = 2.0 g/min.
[3] n.d. not detected.
[a,b,c,d] Means in the same row in columns 1 and 2 with the same letter are not significantly different at p>0.05.
[e,f,g] Means in the same row in columns 3 and 4 with the same letter are not significantly different at p>0.05.

TABLE 2.3. Effect of extraction conditions on the SC-CO_2/EtOH extracts of canola flakes at a CO_2 flow rate of 5.4 g/min.

Pressure (MPa)	55.2	55.2	55.2	41.1	41.1
Temperature (°C)	45	55	70	45	55
Oil content of flakes (w/w %)	13	12	14	11	12
Ethanol % in SC-CO_2 (mole %)	9.5	10.3	10.1	9.2	9.1
Amount of extract (g)	1.5^{c}	2.3^{ab}	2.7^{a}	2.0^{b}	1.8^{bc}
Phospholipid content of extracts (w/w %)	4.0^{a}	1.3^{b}	5.4^{a}	4.4^{a}	5.1^{a}

[a,b,c,d] Means in the same row with the same letter are not significantly different at $p>0.05$.

TABLE 2.4. Effect of ethanol concentration in SC-CO_2 on the SC-CO_2/EtOH extracts of canola meal at 55.2 MPa and 70°C and CO_2 flow rate of 4.5±0.4 g/min.

Ethanol % in SC-CO_2 (mole %)	5.0	9.0
Amount of extract (g)	0.8^a	1.2^b
Phospholipid content of extracts (w/w %)	0.4^a	2.9^b

[a,b] Means in the same row with the same letter are not significantly different at $p>0.05$.

TABLE 2.5. Effect of temperature on the amount and PL concentration of the meal extracts obtained with SC-CO_2/7.5±0.5% EtOH mixture at 55.2 MPa and CO_2 flow rate of 6.7±0.4 g/min. Samples were soaked with 50 mL of ethanol under 55.2 MPa for 90 min. prior to extraction.

Temperature (°C)	45	55	70
Amount of extract (g)	1.5[a]	1.8[a]	1.9[a]
Phospholipid content of extract (w/w %)	28.0[a]	34.5[a]	38.6[a]

[a] Means in the same row with the same letter are not significantly different at $p>0.05$.

TABLE 2.6. Effect of temperature and pressure on the SC-CO_2/13±2% EtOH extracts of canola acetone insoluble fraction.

Pressure (MPa)	55.2	55.2	55.2	41.1	20.7
Temperature (°C)	45	55	70	70	70
Phospholipid content of extracts (w/w %)	41.7^a	3.5^c	6.0^c	20.7^b	16.5^b
Phosphatidylcholine content of extracts (area %)[1]	89^a	24^b	48^b	82^a	84^a

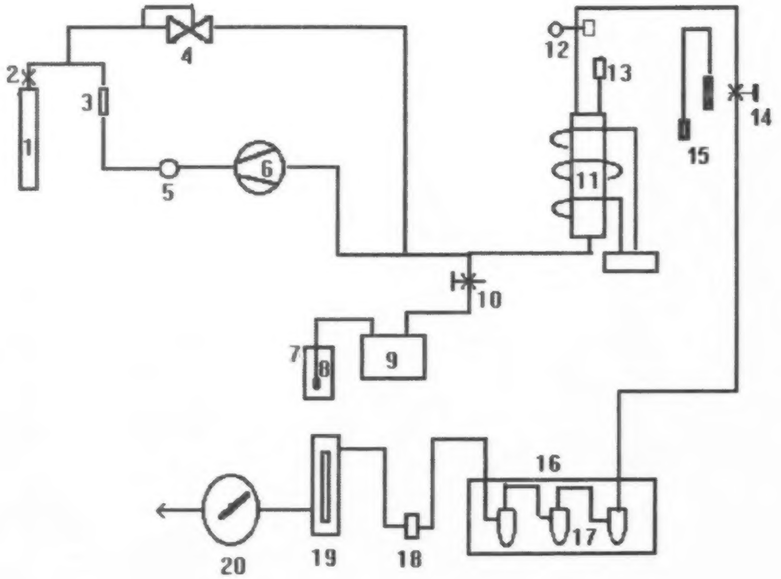
[1] Based on the total Iatroscan peak area of all phospholipid fractions.
[a,b,c]Means in the same row with the same letter are not significantly different at p>0.05.

TABLE 2.7. Effect of the amount of ethanol used for soaking and ethanol percentage in the SC-CO_2 during the extraction period on the acetone insolubles extracts. Samples were soaked at 55.2 MPa and 70°C for 60 min prior to extraction.

EtOH in SC-CO_2 (mole %)	6.5			13.0		
EtOH used in soaking (mL)	20	40	60	20	40	60
Amount of extract (g)	0.25^a	0.40^a	0.50^a	0.30^a	0.40^a	0.50^a
Phospholipid content of extracts (w/w %)	7^c	29^b	50^a	9^c	26^b	32^b
Phosphatidylcholine content of extracts (area %)[1]	66^a	85^a	90^a	73^a	86^a	83^a

[1] Based on the total Iatroscan peak area of all phospholipid fractions.

[a,b,c] Means in the same row with the same letter are not significantly different at p>0.05.



1-CO_2 tank
2-valve
3-CO_2 filter
4-pressure regulator
5-pressure gauge
6-compressor
7-ethanol reservoir
8-filter
9-ethanol pump
10-valve
11-extractor
12-extractor temperature controller
13-rupture disc
14-depressurization valve
15-temperature controller
16-cooling bath
17-collection tubes
18-silica trap
19-flow indicator
20-flow totalizer

FIGURE 2.1. Flow diagram of the SCFE unit.

FIGURE 2.2. Sample material balance and recovery calculations.

(a) Canola Flakes
45 g sample
43% oil⇒ 19.35 g oil
3.7% PL⇒ 0.72 g PL
⇓

SC-CO$_2$ extraction
(70°C, 55.2MPa,
SC-CO$_2$/10.1% EtOH))

2.7 g extract
5.4% PL ⇒ 0.15 g PL
PL recovery ⇒ 20.8%

(b) Canola Meal
65 g Sample
8% oil ⇒ 5.2 g oil
46% PL ⇒ 2.4 g PL
41.5% PC ⇒ 0.99 g PC
⇓

SC-CO$_2$ extraction
(70°C, 55.2MPa, 50 mL EtOH for soaking,
SC-CO$_2$/7.5% EtOH)

1.9 g extract
38.6% PL ⇒ 0.73 g PL
73% PC ⇒ 0.54 g PC
PL recovery ⇒ 30.4%
PC recovery ⇒ 54.5%

(c) Crude canola lecithin
100 g crude lecithin
46.85% PL ⇒ 46.9 g PL
39.6% PC ⇒ 18.6 g PC
⇓

Acetone insolubles
34 g AI
72% PL ⇒ 24.5 g PL
41.6% PC ⇒ 10.2 g PC

SC-CO$_2$ extraction
(without soaking)
(45°C, 55.2MPa,
SC-CO$_2$ /13% EtOH)

1.02 g extract
42% PL ⇒ 0.43 g PL
89% PC ⇒ 0.38 g PC

Recovery based on AI
PL ⇒ 1.75%
PC ⇒ 3.75%
Recovery based on crude lecithin
PL ⇒ 0.91%
PC ⇒ 2.06%

SC-CO$_2$ extraction
(60 mL EtOH for soaking)
(70°C, 55.2MPa,
SC-CO$_2$ /6.5% EtOH)

0.85 g extract
50% PL ⇒ 0.43 g PL
90% PC ⇒ 0.38 g PC

Recovery based on AI
PL ⇒ 1.74%
PC ⇒ 3.76%
Recovery based on crude lecithin
PL ⇒ 0.91%
PC ⇒ 2.06%

3. EFFECT OF EXTRACTION CONDITIONS AND MOISTURE CONTENT OF CANOLA FLAKES ON THE LIPID COMPOSITION OF SUPERCRITICAL CO_2 EXTRACTS[1]

3.1. INTRODUCTION

Moisture content of biological materials is an important factor affecting supercritical fluid extraction (SCFE) and mass transfer kinetics. Moisture content determines the surface structure and activity of the materials (Stahl et al., 1988), e.g. very dry oil seeds have more affinity to oil. High moisture content of the sample acts as a barrier to diffusion of supercritical CO_2 (SC-CO_2) into the matrix as well as diffusion of oil out of the matrix, thus, reducing the SC-CO_2-sample contact. Selectivity of SCFE for the desired compounds is influenced by the level of moisture. Selective extraction of alkaloids from plants, such as caffeine from whole raw beans (Zosel, 1981) and nicotine from crude tobacco (Roselius and Hubert, 1979) with SC-CO_2 require soaking of feed material with water. A Japanese patent (Q.P. Corp., 1987) disclosed that in the presence of water in fat as in butter, cholesterol solubility in supercritical carbon dioxide (SC-CO_2) increased up to 20.0 MPa then decreased with further pressure increase. As well, solubilities of polar essential oil components in SC-CO_2 improved when the moisture content of the samples was increased up to 20% (Stahl and Gerard,

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[1] A version of this chapter is submitted to Journal of Food Science for publication.

1985; Gopalakrishnan and Narayanan, 1991). However, high moisture levels in a sample might have a negative effect on the SCFE of lipids. King et al. (1989) reported that yield of fat extracted with SC-CO_2 increased with decreasing moisture content (~2% w/w) of the meat samples. In this case, water has two opposing effects: it increases fat removal by breaking polar lipid bonds while decreasing the fat removal by inhibiting SC-CO_2-lipid phase contact (Castera, 1994). Selectivity of supercritical fluids (SCF) can also be modified by the addition of a polar component such as water. Saturation of SC-CO_2 with water to increase solvent polarity was applied to essential oil extraction (Stahl and Gerard, 1985).

Swelling of plant materials in the solvent is quite significant for extraction since it widens the cell capillaries increasing porosity and, consequently, improving diffusion. This phenomenon reduces the extraction time and may even make an extraction possible. Solubility of gases in the cell plasma and the elasticity of cell membranes are affected by the moisture content. Varying moisture content of soybean (3-12%) (Snyder et al., 1984), dry-milled corn (3.5-8%) (Christianson et al., 1984) and lupine seed (2.1-24.2%) (Von Stahl et al., 1981) did not affect the SC-CO_2 extractability of oil. However, water does play some part by increasing the permeability of the cell membrane through its swelling effect, hence improving the accessibility of the oil. Eldridge et al. (1986) showed that presence of moisture up to 15% in the soybeans caused denaturation of lipoxygenase with an increase in flavor score but a decrease in nitrogen solubility index.

Moisture had no effect on the FFA or unsaponifiable contents of the SC-CO_2 extracted soybean oil (Snyder et al., 1984).

A portion of the water present in the samples is co-extracted with the oil by SC-CO_2 (Eggers and Stein, 1984; Snyder et al., 1984; Chao et al., 1991). Water co-extraction with rapeseed oil was independent of pressure and feed composition; however, a large water fraction in proportion to the amount of oil was obtained towards the end of extraction (Eggers and Stein, 1984). Examination of soybean meal after SC-CO_2 extraction by Snyder et al. (1984) showed that moisture content increased steadily within the extraction cell in the direction of SC-CO_2 flow. Extracted water was minimal and was observed only during the final stages of extraction. Co-extraction of water is governed by the solubility of water in SC-CO_2, which was reported to be 2-4 mg/g CO_2 at pressures <4.0 MPa, 50°C (Coan and King, 1971). As pressure was increased, water solubility crossed a minimum at about 6.0 MPa then reached asymptotically to a limiting value of about 3 mg water/g CO_2 at 20.0 MPa and 50°C (Wiebe, 1941). Water solubility in CO_2 increases with temperature due to an increase in its vapor pressure (Won, 1983). Model systems consisting of pure solute/SC-CO_2/water have been studied and their behaviour was described using thermodynamic modelling (Wiebe, 1941; Coan and King, 1971; Evelein et al., 1976; Won, 1983; Takishima et al., 1986; Inomata et al., 1989). However, extrapolation of these results to the actual food or other biological systems is difficult due to complex nature of biological systems. Therefore, every system has to be studied individually.

Conventional hexane extraction of canola involves preheating followed by cooking the canola flakes to inactivate enzymes and to rupture the cell structure in order to improve extraction efficiency. Although SC-CO_2 extraction of canola oil was reported (Fattori et al., 1988; Temelli, 1992), further research is needed to optimize all processing parameters, including moisture content and the extent of heat treatment necessary prior to extraction. Thus, the objective of this study was to investigate the effect of moisture content and heat pre-treatment of canola flakes, as well as extraction conditions, such as temperature and pressure on the yields and lipid composition of SC-CO_2 extracts.

3.2. MATERIALS AND METHODS

Flaked canola (*Brassica napus* and *Brassica campestris*) seeds were obtained from Canamera Inc., Fort Saskatchewan, AB, and were kept below -30°C until used. Canola seeds were flaked commercially using a series of roller mills. Canola samples, obtained before and after the conventional cooking process, were referred to as "preheated" and "cooked", respectively. Canola flakes were first preheated up to 40°C in less than 5 min, followed by cooking at 90°C for 15 min.

Moisture content of the cooked canola flakes was modified by adding 0, 4.5 or 7.0 mL deionized water to 45 g of sample, followed by overnight tempering in the refrigerator (4°C) prior to each extraction. Moisture modification of the preheated canola samples was carried out similar to cooked canola samples except that only 0 or 7 mL water was added. Unmodified samples were referred to as "original" preheated or cooked canola. Moisture content of the samples just before and after the SC-CO_2 extraction was determined according to the AACC Method 44-31 (AACC, 1969) and reported on dry and oil-free basis. Water activity of the starting materials was measured with AquaLab CX-2 (Decagon Devices Inc., Pullman, WA) which uses the chilled-mirror dewpoint technique. Oil content of the flaked canola seeds was determined according to the AOCS Official method Ac 3-44 (AOCS, 1990).

Supercritical fluid extraction

A laboratory scale SCFE unit (Newport Scientific Inc., Jessup MD) as described in Chapter 2, was used for this study. The extractor cell was loaded with 35 g canola flakes for each experiment. SCFE experiments were carried out at 35-75°C and 20.7-62.0 MPa for 3 h, at a CO_2 flow rate of 2.5±0.5 g/min.

Triglyceride analysis

Separation of lipid components into FFA, tri-, di- and monoglycerides and quantification based on the peak areas were carried out by an Iatroscan TH-10 Mark II analyzer (T.M.A. Scientific Supply, Mississauga, ON) equipped with precoated silica gel chromarods SIII (Scientific Products Equipment Limited, Concord, ON). The chromarods were developed for 60 min in a solvent system of benzene: chloroform: acetic acid: methanol (50:10:0.2:0.3). Conditions for scanning of chromarods and peak integration were described in Chapter 2. Lipid standards (Sigma Chemical Co., St. Louis, MO); oleic acid, mono-, di- and triolein were used for peak identification. Lipid standards were kept at -80°C. Analyses were performed in triplicate for each sample.

Total free fatty acid content

Total free fatty acid content of SC-CO_2 extracts was determined according to Lowry and Tinsley (1976). A standard curve was prepared with oleic acid for each batch of analyses.

Free fatty acid composition

Extraction of FFA from SC-CO_2 extracts was carried out according to May and Hume (1993). Samples were stored at -80°C until analysis. Individual fatty acids were separated on a capillary gas chromatograph (Model 8320, Perkin Elmer Ltd., Norwalk, CT) equipped with a flame ionization detector and a recording integrator. A fused silica polar capillary column (30 m X 0.32 mm I.D., 0.25 µm df Stabilwax-DA, Crossbond-Carbowax, Restek Inc., Bellefonte, PA) was used for these analyses. Helium was used as the carrier gas. The injector and detector were kept at 250°C. The column pressure was 50 kPa. The oven temperature was held at 200°C for 2 min, followed by heating to 250°C at a rate of 5°C/min and kept at 250°C for 40 min. Individual FFA peaks were identified by comparison to FFA standards obtained from Mandel Scientific (Guelph, ON). Four injections were made for each sample. Means of area percentages were reported.

Statistical analysis

All extraction runs and analyses of each extract were carried out in duplicate, unless otherwise stated, in randomized order and means were reported. Analysis of variance of the results was performed using General Linear Model procedure of SAS

Statistical Software, Version 6 (SAS, 1989). Multiple comparison of the means were carried out by LSD (Least Significant Difference) test at $\alpha = 0.05$ level.

3.3. RESULTS AND DISCUSSION

Extract amount

Analysis of the starting materials is given in Table 3.1. Amount of extract collected during the SC-CO_2 extraction of cooked and preheated canola flakes at various moisture levels is presented in Tables 3.2 and 3.3, respectively. Results of the analysis of variance and multiple comparison of means for the main effects of pressure, temperature, heat pretreatment and moisture content are shown in Table 3.4. Amount of extract collected increased significantly ($p<0.05$) with increasing temperature and pressure. The temperature-pressure interaction effect on the amount of extract was significant ($p<0.05$), since at 20.7 MPa the amount of extract decreased slightly with increasing temperature. The decrease in extract amount with increasing temperature at low pressures is due to the crossover behaviour of the isotherms which was reported previously (Friedrich, 1984; Fattori et al., 1988; Temelli, 1992). For original cooked canola flakes, the highest amount of extract (5.5 g) was obtained at 62.0 MPa and 75°C, which corresponded to a CO_2 loading level of 12.2 mg oil/g CO_2. Amount of extract recovered from the cooked canola flakes was slightly higher than that for the preheated canola samples, however, the effect of heat treatment was not significant ($p>0.05$). Although the flaking process ruptures the cell membranes to a certain degree, the cooking process completes the destruction of the cell membranes facilitating better contact between the solvent and oil. Even though the amount of extract showed an increasing trend with moisture content for preheated samples, the effect of the moisture level in feed material on the amount of SC-CO_2 extract was not significant ($p>0.05$) (Table 3.4). Slight increase in extract amount for preheated canola flakes with increasing moisture content might be due to swelling of the samples which widens the cell capillaries, increasing porosity and improving contact between the solvent and oil. Swelling effects were more pronounced in the preheated samples compared to the cooked samples, since cell membranes were already ruptured during the cooking process.

Moisture effect

In this study, the highest moisture content of the feed samples examined was targeted to be ~20% (as is basis, which corresponds to ~50% on dry and oil-free basis) to approach the natural moisture content of oil seeds at harvest. Moisture analysis results of the canola flakes before and after SC-CO_2 extraction were reported on dry and oil-free basis to facilitate better comparison among samples with different moisture and oil contents (Tables 3.1-4).

Moisture content of the original preheated canola flakes (15.9%) was slightly higher than that of the cooked flakes (12.7%). Moisture content of residual flakes after extraction was lower than that of the feed material for all samples and conditions studied, indicating that some water was co-extracted along with canola oil during SC-

CO_2 extraction. Similar results were obtained with soybean and rapeseed (Eggers and Stein, 1984; Snyder et al., 1984). Moisture loss from original preheated canola flakes was slightly higher than from the original cooked canola flakes as shown in Table 3.3 and Table 3.2, respectively. This can be attributed to the higher free moisture content of the preheated samples as reflected in their water activity levels (Table 3.1). Amount of moisture lost from the samples during SC-CO_2 extraction significantly ($p<0.05$) increased with temperature and moisture content of feed material and decreased with pressure. Highest moisture loss was achieved at 20.7 MPa and 75°C. These results can be explained by the solubility of water in SC-CO_2 which is higher at lower pressures and solubility increases with temperature due to an increase in its vapor pressure (Won, 1983). Heat pretreatment, pressure, temperature, moisture content of feed material and pressure-temperature interaction effects on the final moisture content of residual meal were significant at $p<0.05$ level.

SC-CO_2 extracts obtained from cooked canola samples at high pressures were lighter in colour and clear compared to those at lower pressures. Similar observations were reported by Stahl et al. (1981). Friedrich and List (1982) reported that soybean SC-CO_2 extracts obtained at high temperatures were cloudy and thicker than the extracts collected at a lower temperature and at the same pressure. In our study, the effect of pressure on the appearance of the extracts seemed to be more pronounced than that of temperature. Extracts obtained at 20.7 MPa and 75°C were mainly water with a milky appearance. Milky droplets were observed in the oil even at high pressures, when initial moisture content of samples was increased. Consistency and cloudiness of the extracts seemed to be correlated with the moisture loss from the feed material. Color of the SC-CO_2 extracts got darker and more cloudy with increasing moisture loss from the feed material during extraction. It was not possible to quantify the amount of water in the SC-CO_2 extracts due to very small sample size. However, quantification of the co-extracted water along with oil at different SC-CO_2 extraction conditions and moisture levels should be considered for further research. Oil extracted from preheated canola was darker than that from cooked canola samples which might be due to enzymatic reactions. A light-colored oil product was obtained from preheated canola only at 41.4, 20.7 MPa and 35°C.

FFA content of extracts

Total FFA contents of the feed material and SC-CO_2 extracts are given in Tables 3.1-4. FFA content of the hexane-extracted oil from original preheated canola flakes (1.6%) was lower than that from the original cooked canola samples (2.9%) which may be due to lipid hydrolysis reactions occurring during the cooking process. FFA contents of the SC-CO_2 extracts of original cooked canola flakes (Table 3.2) at 62.0 MPa and 35-75°C were similar to that of hexane extracts. Similarly, Friedrich and Pryde (1984) showed that at 34.0 MPa and 50°C, FFA content of the SC-CO_2 extracted soybean oil was comparable to that of the hexane extracts. However, our experiments showed that FFA content of the extracts increased significantly ($p<0.05$) with decreasing pressure (Table 3.4). Similar trends were observed for extracts of cooked canola flakes with higher moisture content (Table 3.2) and preheated canola samples (Table 3.3). Increased FFA concentration of extracts obtained at low pressures is due to lower molecular weight of FFA compared to TG. As pressure is decreased solubility of TG in SC-CO_2 decreases with a reduction in CO_2 density. Therefore, selectivity of SC-CO_2 is shifted towards low molecular weight FFA. It was also shown that FFA became concentrated in the earlier oil fractions during the SC-CO_2 extraction of soybeans (Friedrich and Pryde, 1984). Extraction time was limited to 3 h in this study. At low pressures, oil recovery was only ~10%, hence FFA became concentrated in the small amount of extract. FFA contents of the extracts obtained at different temperatures were similar ($p>0.05$). Heat treatment had a significant effect and FFA content was higher ($p<0.05$) for cooked canola extracts, which is due to hydrolysis reactions taking place during the cooking process. FFA content of the extracts obtained from cooked canola flakes with 29.9 and 37.8% moisture at 20.7 MPa/ 75°C was quite low due to higher water levels in the extracts which had a dilution effect on the extract FFA composition.

Triglyceride content of extracts

TG analysis results of cooked canola extracts are given in Table 3.5. At 62.0 and 41.4 MPa, TG content of the SC-CO_2 extracts was similar to that of the hexane-extracted oil (Table 3.1). However, at 20.7 MPa, TG content of the samples decreased with increasing temperature and moisture content. These results correlate with FFA results. Those extracts with a higher FFA content had a low TG content.

FFA composition of the extracts

FFA compositions of cooked canola extracts are presented in Table 3.6. Fatty acid composition of FFA fractions of canola oil obtained with SC-CO_2 was different from that of the previously reported overall fatty acid composition of canola oil (Temelli, 1992). Oleic and linoleic acids had the highest concentration in both canola oil and FFA fraction. However, palmitic acid (16:0) concentration in the FFA fraction was much higher than that reported in the total canola oil extract. This might be due to the smaller molecular weight of palmitic acid. Concentration of linolenic acid (18:3) was similar to the overall values.

3.4. CONCLUSIONS

Amount of SC-CO_2 extracts increased with pressure for both cooked and preheated canola. With an increase in temperature, the extract yield increased at high pressures, but decreased at 20.7 MPa due to crossover of solubility isotherms. Color and turbidity of the extracts were affected by extraction conditions. Since some water is co-extracted with canola oil, quantification of water in the SC-CO_2 extracts is essential for the determination of optimum oil yield conditions. Addition of a second collection unit to separate water from the oil extract would be beneficial. Moisture modification of the feed material did not improve SC-CO_2 canola extract properties. Although cooking process improved appearance of SC-CO_2 extracts, FFA contents of the extracts were higher than that of the preheated samples. Further characterization including polar lipid composition, keeping quality, nonsaponifiable content and sensory properties of the SC-CO_2 canola extracts obtained at different extraction conditions and the effect of degree of extraction on the lipid composition should be considered for further research.

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TABLE 3.1. Properties of feed materials for SC-CO_2 extraction.

Sample	Cooked canola	Preheated canola
Oil content (%, w/w)	44.0	40.4
Moisture content (%, w/w)	6.3[1]	8.2[1]
	12.7[2]	15.9[2]
Water activity (at 27°C)	0.584	0.661
FFA content (%, w/w)[2]	2.9	1.6
Triglyceride content (Iatroscan area %)[3]	98.6	98.4

[1] wet basis
[2] dry and oil-free basis
[3] hexane extracted oil

TABLE 3.2. Effect of SC-CO_2 extraction conditions and moisture content of the cooked canola samples on the extracts.

Cooked canola		Pressure (MPa)								
Moisture before extraction (%, w/w)[1]	Temperature (°C)	20.7	41.4	62.0	20.7	41.4	62.0	20.7	41.4	62.0
		Extract amount (g)			Moisture after extraction (%, w/w)[1]			FFA content of the extracts (%, w/w)		
12.7	35	1.0	2.2	3.3	10.9	11.7	11.5	5.6	3.5	2.8
	55	0.8	2.4	4.3	9.8	11.1	11.1	8.1	3.4	2.5
	75	0.6	3.2	5.5	8.9	9.0	11.2	7.8	3.6	2.4
29.9	35	1.2	2.2	2.8	27.6	29.0	27.9	5.5	3.0	2.9
	55	1.1	2.5	5.0	25.2	28.7	27.1	4.6	2.9	2.2
	75	0.8	3.5	7.1	24.7	25.3	26.0	1.7	2.8	2.2
37.8	35	1.1	2.0	3.0	36.7	36.5	35.6	6.3	3.7	3.1
	55	1.2	3.8	4.4	35.7	34.7	36.6	5.2	2.6	2.5
	75	1.0	4.0	6.0	29.2	32.7	36.3	1.5	3.0	2.4

[1] dry and oil-free basis

TABLE 3.3. Effect of SC-CO_2 extraction conditions and moisture content of the preheated canola samples on the extracts.

Preheated canola		Pressure (MPa)								
Moisture before extraction (%, w/w)[1]	Temperature (°C)	20.7	41.4	62.0	20.7	41.4	62.0	20.7	41.4	62.0
		Extract amount (g)			Moisture after extraction (%, w/w)[1]			FFA content of the extracts (%, w/w)		
15.9	35	0.9	1.8	2.7	13.8	14.1	14.2	2.3	1.3	1.3
	55	1.0	2.4	3.6	11.9	13.9	14.5	3.5	1.7	1.4
	75	0.8	2.8	5.4	11.0	12.2	14.0	3.6	2.5	1.8
42.5	35	1.2	2.3	2.9	38.9	40.3	41.1	3.4	2.0	1.8
	55	0.9	2.8	5.1	40.0	39.7	39.0	5.6	1.9	1.5
	75	0.7	3.9	6.5	35.6	34.6	40.5	5.8	2.4	2.1

[1] dry and oil-free basis

TABLE 3.4. Comparison of the means for the main effects of pressure, temperature, heat treatment and moisture on the extract amount, moisture after extraction and free fatty acid content of extracts.

Variable	Extract amount (g)	Moisture after extraction (%, w/w)	FFA content of extracts (%, w/w)
Pressure (MPa)			
20.7	0.9^{a}	24.0^{a}	4.7^{a}
41.4	2.7^{b}	24.9^{b}	2.7^{b}
62.0	4.5^{c}	25.8^{c}	2.1^{c}
Temperature (°C)			
35	2.0^{a}	26.0^{a}	3.1^{a}
55	2.9^{b}	25.3^{a}	3.2^{a}
75	3.7^{c}	23.4^{b}	3.0^{a}
Heat treatment			
Cooked	3.0^{a}	24.1^{b}	3.6^{b}
Preheated	2.7^{a}	26.0^{a}	2.4^{a}
Moisture before extraction (%, w/w)			
Cooked			
12.7	2.9^{a}	10.6^{e}	4.3^{a}
29.9	3.0^{a}	26.8^{c}	3.1^{b}
37.8	3.1^{a}	34.9^{b}	3.4^{ab}
Preheated			
15.9	2.6^{a}	13.3^{d}	2.1^{c}
42.5	2.9^{a}	38.8^{a}	2.9^{bc}

[1] dry and oil-free basis

[a,b,c] Means in each column within each section of pressure, temperature, heat treatment and moisture before extraction with the same letter are not significantly different at $p>0.05$.

TABLE 3.5. Triglyceride content of SC-CO_2 extracts of cooked canola flakes.

Cooked Canola		Pressure (MPa)		
Moisture before extraction (%, w/w)[2]	Temperature (°C)	20.7	41.4	62.0
		Triglyceride Concentration (%[1])		
12.7	35	97.5	97.1	97.0
	55	95.7	98.3	98.7
	75	n.d.[3]	97.8	98.7
29.9	35	96.3	98.3	97.7
	55	95.3	97.2	98.2
	75	93.1	97.8	97.7
37.8	35	96.9	97.8	98.3
	55	96.0	97.8	98.3
	75	91.9	98.1	97.5

[1] Iatroscan area %

[2] dry and oil-free basis

[3] n.d. not determined (due to small sample size)

TABLE 3.6. Fatty acid composition (GC area %) of FFA acid fraction of cooked canola SC-CO_2 extracts at 75°C.

Moisture before extraction (%, w/w)[1]	12.7		29.9		37.8	
Pressure (MPa)	41.4	62.0	41.4	62.0	41.4	62.0
Palmitic (16:0)	19.0	14.3	14.9	15.9	14.6	13.3
Stearic (18:0)	5.0	5.0	3.3	5.1	4.4	4.3
Oleic (18:1)	40.9	40.8	41.0	39.4	41.2	41.6
Linoleic (18:2)	28.1	32.3	33.1	32.1	31.9	30.8
Linolenic (18:3)	7.1	7.6	7.6	7.5	7.9	9.9

[1] dry and oil-free basis

4. EFFECT OF SC-CO_2 ON MYROSINASE ACTIVITY AND GLUCOSINOLATE DEGRADATION IN CANOLA[1]

4.1. INTRODUCTION

Glucosinolates are found mainly in the *Cruciferae* crops, e.g cabbage, turnips, radishes, mustard, rapeseed and canola. Glucosinolates contain a β-thioglucose unit, a side chain R group and a sulphonated oxime moiety (Fig. 4.1). These sulphur containing compounds contribute to the characteristic pungent odor and bitter taste of the cruciferae plants. Up to date, more than 100 glucosinolates have been identified. In canola, 12 glucosinolate compounds were detected. A list of the major glucosinolates present in canola is given in Figure 4.1 (Shahidi and Gabon, 1989). Intact glucosinolates are not toxic, however, their hydrolytic products were associated with goitrogenicity, toxicity and antinutritional effects in animals (Vermorel et al., 1988). The medicinal properties of the volatile, pungent oils from *Cruciferae* plants have been known for many centuries (McDanell et al., 1988). By the end of the last century, it was known that volatile oils were isothiocyanates formed upon crushing the *Cruciferae* plant in water (McDanell et al., 1988). Recently, hydrolysis products of indole glucosinolates have received special attention due to their anti-carcinogenic functions. There is some evidence that administration of indole glucosinolates during the initiation period of carcinogenesis may cause inhibition of tumor production while administration during the promotion period may enhance the carcinogenic effect (McDanell et al., 1988).

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[1] A version of this chapter is to be submitted to Journal of Agricultural and Food Chemistry for publication.

The presence of glucosinolates in rapeseed has been a major limitation for its use as animal feed. Several methods have been developed for removing the undesirable glucosinolates and their hydrolysis products from rapeseed meal. Hydrothermal treatment of intact seeds in sodium sulphate at 100°C (Mothadi-Nia et al., 1986), extraction of ground seeds with a two-phase solvent system consisting of ammonia-methanol or ammonia-water and hexane (Diosady et al., 1985) and soaking of intact seeds with citric acid or ammonium carbamate (Schwenke et al., 1990) were some of the methods investigated. Procedures designed to extract glucosinolates from canola flour were not efficient (Sosulski and Dabrowski, 1984). Plant breeding seems to be the primary technique used to overcome the glucosinolate problem during the development of canola varieties from rapeseed. Indeed, plant breeding techniques have been very successful in lowering the glucosinolate contents from 100-205 µmol/g (traditional rapeseed) to <30 µmol/g (canola).

Although glucosinolates are mainly hydrolysed by enzyme reactions, thermal degradation of various glucosinolates was also reported (MacLeod et al., 1981; Sosulski and Dabrowski, 1984; Goronowitz et al., 1978). However, non-enzymatic thermal degradation of glucosinolates requires higher temperatures (>100°C) than enzymatic degradation. Conventional canola processing involves heat treatments to condition the seeds for improved oil extraction, to inactivate myrosinase enzyme, to remove solvent and to dry the meal. Seeds are flaked, preheated at 20-50°C and cooked at 80-95°C for 15-30 min. Generally, water or steam is not added to flakes during the cooking process to avoid acceleration of enzymatic glucosinolate hydrolysis (Campbell, 1984). However, crushing brings myrosinase and glucosinolates in close contact and some hydrolysis is inevitable until the enzyme is inactivated. Hydrolytic products, which are oil soluble, enter the oil during extraction. Sulphur compounds in oil cause catalyst poisoning during hydrogenation and unpleasant odors in heated oil (Daun and Hougen, 1976). Campbell and Slominski (1990) showed that decomposition of indole glucosinolates before the desolventization stage was minor during canola processing, while major glucosinolate decomposition occurred during desolventization of meal. Effect of meal drying on the glucosinolate degradation was minimal.

Myrosinase (thioglucoside glucohydrolase, E.C.3.2.1.) is present in all plants containing glucosinolates (Fenwick et al., 1983) and catalyzes the hydrolysis of glucosinolates. Myrosinase is located outside the myrosin cells (protein accumulating idioblasts) and has the tendency to adhere to membrane surfaces (Höglund et al., 1991), whereas glucosinolates are found in the myrosin cells. Therefore, myrosinase and glucosinolates do not come in contact unless the seed tissues are damaged. Myrosinase specifically cleaves the thioglucosidic bond and the resulting aglucone spontaneously undergoes a Lossen rearrangement to produce isothiocyanate as shown in Figure 4.2 (MacLeod and Rossiter, 1986). Enzymic hydrolysis of glucosinolates at acidic pH leads predominantly to nitrile whereas isothiocyanate is the main product at neutral pH (MacLeod and Rossiter, 1986).

Kozlowska et al. (1983) studied inactivation of rapeseed myrosinase and showed that enzyme activity was lost rapidly above 65°C in the crude extract while inactivation in intact and flaked seeds started after 5 min incubation at 90-100°C and 90-100% relative humidity. Myrosinase inactivation was easier in flaked seeds than in intact seeds. No glucosinolate degradation was observed in intact rapeseed. However, glucosinolates were substantially hydrolysed in flaked seeds during myrosinase inactivation since the enzyme was already in contact with the substrate. Therefore, Kozlowska et al. (1983) proposed that the rapeseed processing industry should consider inactivation of myrosinase in intact seed before rather than after the flaking process. In another study (Appelqvist and Josefsson, 1967), a small loss of myrosinase activity was observed after heat treatment of rapeseed at 90°C and 4% moisture content for 15 min, while the same heat treatment at 6-8% moisture content effectively inactivated the enzyme. Similarly, Eapen et al. (1968) showed that dry heat treatment (104°C for 30 min) did not decrease myrosinase activity, while steam blanching (5-30 min) was effective in completely inactivating the enzyme. Soaking of *B. napus* and *B. campestris* in boiling water for 1.5 and 1 min, respectively, resulted in complete myrosinase inactivation (Eapen et al., 1968). Microwave inactivation of myrosinase in canola seeds was studied by Owusu-Ansah and Marianchuk (1991). They concluded that the exposure time needed for enzyme inactivation at a specific power was moisture-dependent. More recently, McCurdy (1992) reported that infrared processing of canola fines at 78°C had little effect on myrosinase activity, while processing at 105°C resulted in high level of enzyme inactivation. This treatment was more effective in canola fine screenings than in canola seeds, which was attributed to the higher moisture content of the screenings (9.9%) than the seeds (5.3%) (McCurdy, 1992). Utilization of *Cruciferae* plants containing intact glucosinolates as animal feed following myrosinase inactivation may not be safe, since myrosinase present in other plants and in certain intestinal organisms could hydrolyse ingested glucosinolates in the intestinal tract. Thus as Slinger (1977) indicated, the ultimate solution for glucosinolate degradation problem is to breed a "zero" glucosinolate variety rapeseed.

Effect of supercritical carbon dioxide (SC-CO_2) on enzyme activity varies with processing conditions and the type of protein. Studies with soy protein showed that lipoxygenase was inactivated during SC-CO_2 extraction of oil at 73-85.4 MPa and 80-100°C (Eldridge et al., 1986). Christianson and coworkers (1984) reported that peroxidase activity was reduced tenfold during SC-CO_2 extraction of corn germ at 34.5-55.2 MPa and 50°C. Pectinesterase in single strength orange juice could be inactivated with SC-CO_2 at 40°C and 31 MPa (Balaban et al., 1991). Effect of SC-CO_2 on several commercial enzyme preparations was studied by Taniguchi et al. (1987a) at 20.3 MPa, 35°C for 1 h. All of the enzymes investigated retained over 90% of their initial activity following SC-CO_2 treatment. Presence of ethanol in SC-CO_2 and increasing moisture content of samples were factors improving the effectiveness of the enzyme inactivation.

Taniguchi et al. (1987b) extracted oil from mustard seeds with SC-CO_2 without lowering the myrosinase activity and sinigrin content of the samples.

To date, the effect of SC-CO_2 on the endogenous myrosinase enzyme, its activity and glucosinolate degradation reactions in canola has not been reported. Glucosinolate hydrolysis reactions under SC-CO_2 conditions should be investigated for proper optimization of SC-CO_2 extraction conditions and to determine the extent of seed heat pretreatment necessary prior to oil extraction congruent with evaluation of the quality of extracted oil and residual meal. Therefore, the objective of this study was to examine the effect of SC-CO_2 processing conditions; temperature, pressure, extraction time and moisture content of the samples on the myrosinase activity and glucosinolate hydrolysis in flaked and whole canola seeds.

4.2. MATERIALS AND METHODS

Flaked canola (*B. napus* and *B. campestris*) and whole seeds were obtained from Canamera Inc., Fort Saskatchewan, AB, and kept below -30°C until used. Canola seeds were flaked commercially using a series of roller mills. Canola flake samples were obtained prior to the conventional cooking process and were referred to as "preheated", since they were heated up to 40°C in less than 5 min following flaking. Moisture contents of the canola flakes and whole seeds were modified by adding 7.0 mL deionized water to 45 g of sample followed by overnight tempering in the refrigerator (4°C) prior to each extraction. Unmodified samples were referred to as "original" preheated flakes or seeds. Moisture content of the samples just before the SC-CO_2 extraction was determined according to the AACC Method 44-31 (AACC, 1969) and reported on a basis of w/w %.

SC-CO_2 extraction

A laboratory scale SCFE unit (Newport Scientific Inc., Jessup MD) as described in Chapter 2 was used. The extractor cell was loaded with 35 g canola flakes for each experiment. SC-CO_2 extractions were carried out at 35-75°C and 21.4-62.1 MPa for 3 h. CO_2 flow rate was maintained at 2.5±0.5 g/min. Experimental procedures for whole seeds were similar to that of the flaked seeds except extraction conditions were kept constant at 75°C and 62.1 MPa, while varying the extraction time as 1, 3, and 5 h. Residual samples after SC-CO_2 extraction were kept at -80°C until they were analyzed for their enzyme activity and glucosinolate content.

Enzyme activity

Myrosinase activity was measured according to Owusu-Ansah and Marianchuk (1991) method after modifications as follows: A 10 g canola sample was homogenized in 50 mL chilled acetone (reagent grade Omni solvent, BDH Inc., Toronto, ON) for 2x30 sec using a Kinematica polytron (Brinkman Instruments, Rexdale, ON) on dry ice. The homogenate was filtered and washed with an excess of chilled acetone. The retentate, which is referred to as "acetone powder", was spread out on filter paper and air dried for 1 h at room temperature. One g of acetone powder was re-extracted with 15 mL of cold phosphate buffer (pH 7.0, 0.1 M). Then slurry was centrifuged (Beckman

Instruments Inc., Mississauga, ON) at 28,200xg for 30 min at 4°C and the supernatant was used as the crude enzyme preparation.

Two mL of sinigrin (Sigma Chemical Co., St. Louis, MO) solution (1 mM in phosphate buffer) and 2 mL of crude enzyme solution were mixed on a vortex. The mixture was incubated at 35°C for 1 h, followed by inactivation of the enzyme by boiling for 15 min. After cooling under tap water, 100 μL aliquot was used for glucose analysis according to O-toluidine procedure (glucose kit catalog # 635, Sigma Chemical Co., St. Louis, MO). Glucose content of the enzyme solutions before and after substrate incubation period was determined for each sample. Difference between the glucose contents before and after the incubation was taken as the glucose converted by the enzyme in 1 h. Protein content of the enzyme solutions was determined using phenol reagent method (protein kit catalog # 690-A, Sigma Chemical Co., St. Louis, MO). Enzyme activity was calculated using the following equations:

$$\text{Enzyme activity} = \frac{\mu\text{g glucose converted by the enzyme in 1 h}}{\mu\text{g protein in the enzyme solution x 60 min}} \tag{4.1}$$

$$\text{\% residual activity} = \frac{\text{enzyme activity after SC-CO}_2\text{ extraction}}{\text{enzyme activity before SC-CO}_2\text{ extraction}} \times 100 \tag{4.2}$$

Glucosinolate analyses

Intact glucosinolates in canola samples were isolated and purified on a DEAE ion-exchange column (DeClercq and Daun, 1989).

Extraction

Canola samples (5 g) were heated at 95°C for 15 min for inactivation of enzymes. Four mL of boiling water was added into the ground sample (200 mg) and mixed quickly to reduce cooling and then the mixture was heated at 95°C for 5 min. After cooling and centrifugation at 900xg, the supernatant was transferred into graduated centrifuge tubes containing 150 µL of 0.5 M barium/lead acetate solution. Residue from the first water extraction was re-extracted with 4 mL boiling water for 5 min at 95°C as above. Supernatant from the two extraction steps were combined and the volume was adjusted to 10 mL. The mixture was centrifuged (900xg) and then supernatant was used for glucosinolate purification.

Column preparation

Dry DEAE-Sephadex A-25 (100 mg) in 0.8x4 cm Econo-column (Bio-Rad Labs., Hercules, CA) was allowed to swell in deionized water. Air bubbles were removed by stirring. Prior to sample application, 5 mL of 0.5 N NaOH, followed by 10 mL water to remove excess NaOH, were passed through the column. The eluate was monitored to ensure neutral pH. The resin was changed to acetate form by adding 5 mL of 0.5 M pyridine acetate solution followed by 10 mL water.

Isolation of glucosinolates

Three mL of crude glucosinolate extract were applied to the prepared column. Then, the column was washed with 2x2 mL water, 2x2 mL 3% formic acid and 2x2 mL water, discarding the eluate each time. Glucosinolates were eluted with 2x4.75 mL of 0.3 M K_2SO_4 and volume was adjusted to 10 mL.

Measurement of glucosinolate content

A one mL aliquot of isolated glucosinolates was mixed with 7.0 mL 80% H_2SO_4 and 1.0 mL of 1% thymol in ethanol. The mixture was incubated at 100°C for 60 min, then cooled and mixed. Absorbance was measured against 0.3 M K_2SO_4 at 505 nm using a diode array spectrophotometer (Model 8452A, Hewlett-Packard Ltd., Orangeville, ON). Four blanks and four standards were prepared. Standards consisted of 1.0 mL sinigrin solution (0.3 μmol/mL), 7.0 mL 80% H_2SO_4 and 1.0 mL 1% thymol solution, while blanks were prepared with 1 mL 0.3 M K_2SO_4, 7.0 mL 80% H_2SO_4 and 1.0 mL 1% thymol solution. Glucosinolate content of the canola samples reported as μmol/g dry and oil free sample, were calculated by using the averages of absorbance values for standard and test solutions using the following equations:

$$K=\frac{(A_S-A_B)}{C} \tag{4.3}$$

where, K = absorption factor

A_s = mean absorbance of standards

A_B = mean absorbance of blanks

C_s = concentration of the standards, µmol/mL;

and

$$C_x = \frac{A_x}{K} x \frac{DF}{W} \tag{4.4}$$

where, C_x = concentration of glucosinolates in sample, µmol/g

A_x = absorbance of sample

K = absorption factor from Eq. 4.1.

DF = dilution factor (depending on extraction volume and aliquot sizes)

W = weight of sample, g

Statistical analysis

All SC-CO_2 extractions and analyses of the residue from each SC-CO_2 extraction were carried out in duplicate, unless otherwise stated, in randomized order and means were reported. Analysis of variance of the results was carried out using General Linear Model procedure of SAS Statistical Software, Version 6 (SAS, 1989). Multiple comparison of the means were carried out by LSD (Least Significant Difference) test at $\alpha = 0.05$ level.

4.3. RESULTS AND DISCUSSION

Myrosinase activity

Flaked canola seeds (7.7% moisture) had significantly ($p<0.05$) lower enzyme activity than the whole seeds (8.5% moisture), 2.0 and 2.7 μg glucose/μg protein-min, respectively, which might be due to the flaking and preheating processes.

When canola flakes (7.7% moisture) were exposed to SC-CO_2 at 21.4-62.1 MPa and 35-75°C for 3 h, 85-100% of original myrosinase activity was retained (Table 4.1). The effects of temperature and pressure were not significant ($p>0.05$) and the residual enzyme activities following SC-CO_2 treatment were similar to that in the feed material. These results were similar to those reported for mustard myrosinase activity following SC-CO_2 treatment at 30.0 MPa and 40°C for 3 h (Taniguchi et al., 1987b). To assess the effect of moisture content of canola flakes on the myrosinase activity during SC-CO_2 processing, a series of experiments were performed with flakes containing 19.0% moisture (Fig. 4.3). At lower temperatures, (<55°C), ≥85% of the original enzyme activity was retained in the canola flake residues which was similar ($p>0.05$) to that in the feed material. At 19% moisture level, there was a significant ($p<0.05$) drop in enzyme activity with an increase in temperature up to 75°C at all pressure levels studied (Fig. 4.3).

Highest myrosinase inactivation was achieved at 62.1 MPa and 75°C, where only 10% of the original enzyme activity was retained after 3 h. At 41.1, 21.4 MPa and 75°C residual enzyme activities were 50 and 20% of the original, respectively (Fig. 4.3).

The fact that the residual enzyme activity at 21.4 MPa was significantly ($p<0.05$) lower than that at 41.4 MPa was unexpected. Literature lacks information on the effect of SC-CO_2 treatment on enzyme activity as a function of pressure. However, Temelli et al. (1995) reported increased level of aggregation of sarcoplasmic proteins of Atlantic mackerel with presssure (34-62 MPa) during SC-CO_2 extraction of oil. Another factor contributing to the denaturation of proteins during SC-CO_2 treatment at high moisture levels is the formation of carbonic acid due to the equilibrium reached between CO_2 and water. This equilibrium is shifted towards carbonic acid with pressure. It was not possible to measure the pH of canola flakes during the SC-CO_2 extractions. Further research is needed to understand the mechanism of myrosinase inactivation under combined effects of pressure and temperature at high moisture levels in SC-CO_2 environment.

Time dependence of myrosinase inactivation in whole canola seeds were examined at 62.1 MPa and 75°C, since the highest enzyme inactivation was achieved under these conditions with flaked seeds. After 3 h of SC-CO_2 treatment of whole canola seeds (8.5% moisture) no enzyme inactivation was observed (Fig. 4.4). Ninety three percent of the original myrosinase activity was retained in the whole seeds even after 5 h of SC-CO_2 treatment. However, when moisture content of the whole seeds was modified to 20.5%, there was significant ($p<0.05$) enzyme inactivation (Fig. 4.4). At this moisture level, residual myrosinase activities were 74, 56, and 26% after 1, 3 and 5 h SC-CO_2 treatment of canola seeds, respectively. Comparison of myrosinase inactivation in canola flakes and whole seeds under SC-CO_2 conditions indicates that enzyme inactivation was harder to achieve in whole seeds than flaked seeds. After 3 h of SC-CO_2 processing at 62.1 MPa and 75°C, residual myrosinase activity in canola flakes (7.7% moisture) was 85% (Table 4.1), whereas in whole seeds (8.5% moisture) (Fig. 4.4) no enzyme inactivation was detected. A similar trend was observed at the higher moisture level, but the residual enzyme activity measured in whole seeds, 56% (Fig. 4.4), was significantly ($p<0.05$) higher than that in the flaked seeds, 10% (Fig. 4.3). The same trend was observed during the heat inactivation of rapeseed (Kozlowska et al., 1983).

Glucosinolate content

Glucosinolate content of canola flakes was slightly lower than that of the whole seeds, 9.6 and 10.2 μmol/g, respectively. This result was not unexpected since some enzymatic glucosinolate hydrolysis is inevitable as a result of the flaking process which brings myrosinase into contact with glucosinolates. Comparable glucosinolate levels in canola were reported by Owusu-Ansah and Marianchuk (1991). This level of glucosinolates in canola is well below the Canadian standard of 30 μmol/g meal.

Reduction in glucosinolate content of canola flakes (7.7% moisture) was minimal after SC-CO_2 treatment under the temperature and pressure conditions studied (Table 4.2) despite the fact that there was no significant change in myrosinase activity (Table 4.1). When the moisture content of the flakes was modified to 19.0%, slightly higher glucosinolate degradation was observed at 55, 75°C and 41.1-62.1 MPa. Although enzyme activity was significantly ($p<0.05$) decreased at 75°C (Fig. 4.3) some glucosinolates were hydrolysed during the inactivation process. This might be due to the faster glucosinolate hydrolysis rates than the enzyme inactivation rate at higher temperatures. At the highest temperature studied, 75°C, highest residual enzyme activity was observed at 41.4 MPa, which resulted in the largest glucosinolate degradation.

Figure 4.5 presents glucosinolate degradation in whole seeds with SC-CO_2 extraction time at different moisture levels. At 8.5% moisture level, there was no change in the glucosinolate content up to 3 hours of SC-CO_2 extraction. Glucosinolate degradation reached a significant level ($p<0.05$) during the last two hours of SC-CO_2 extraction. At 20.5% moisture level, there was only a slight decrease in glucosinolate content during the first hour which remained steady upto 5 h (Fig. 4.5). This can be attributed to the retention of original activity at 8.5% moisture level, as opposed to the significantly ($p<0.05$) reduced myrosinase activity of the samples containing 20.5% moisture (Fig. 4.4). Enzyme inactivation in whole seeds with higher moisture content was much faster than that of the lower moisture content seeds (Fig. 4.4). Therefore, seed samples with low moisture level had higher enzyme activity for glucosinolate hydrolysis throughout the SC-CO_2 treatment.

4.4. CONCLUSIONS

Myrosinase inactivation and glucosinolate hydrolysis in canola samples were minimal under SC-CO_2 conditions at low moisture levels. Combined effects of high temperature (75°C), pressure (62.1 MPa) and moisture level (20%) were necessary to achieve 90% reduction in enzyme activity. Myrosinase inactivation in whole canola seed was harder to achieve than that in the flaked samples. Increasing the moisture content of the whole seeds reduced the glucosinolate degradation rate due to low level of residual myrosinase activity. Therefore, in terms of SC-CO_2 processing of canola flakes at low moisture levels, myrosinase inactivation prior to SC-CO_2 extraction may not be necessary.

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TABLE 4.1. Effect of SC-CO_2 extraction temperature and pressure on the myrosinase activity in canola flakes with 7.7% moisture content.

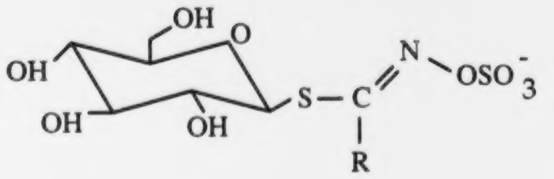
Temperature (°C)	Pressure (MPa)		
	21.4	41.4	62.1
	Enzyme activity (µg glucose/µg protein-min)		
35	1.8[a]	2.0[a]	1.8[a]
55	1.7[a]	1.9[a]	1.9[a]
75	1.7[a]	2.0[a]	1.7[a]

[a] Means with the same letter are not significantly different ($p>0.05$).

TABLE 4.2. Effect of temperature, pressure and moisture on the glucosinolate content of canola flakes during SC-CO_2 treatment.

Temperature (°C)	Moisture content (%,w/w)					
	7.7			19.0		
	Pressure (MPa)					
	21.4	41.4	62.1	21.4	41.4	62.1
	Glucosinolate content (μmol/g)					
35	9.6^{a}	9.3^{ab}	9.5^{ab}	9.6^{a}	9.2^{ab}	9.4^{ab}
55	9.5^{ab}	9.2^{ab}	9.4^{ab}	9.5^{ab}	8.9^{ab}	8.6^{ab}
75	8.9^{ab}	9.1^{ab}	9.4^{ab}	8.9^{ab}	8.4^{b}	8.9^{ab}

[a] Means with the same letter are not significantly different ($p>0.05$).



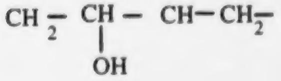
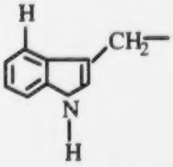
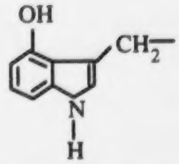
Structure of R group	**Semisystematic name**	**Trivial name**
$CH_2{=}CH{=}CH_2-$	Allylglucosinolate	Sinigrin
$CH_2{=}CH-CH_2-CH_2-$	But-3-enylglucosinolate	Gluconapin
$CH_2{=}CH-CH_2-CH_2-CH_2-$	Pent-4-enylglucosinolate	Glucobrassicanapin
 $CH_2{=}\underset{\overset{\vert}{OH}}{CH}-CH-CH_2-$	(2R)-2-Hydroxybut-3-enylglucosinolate	Progoitrin
	Indol-3-ylmethyl glucosinolate	Glucobrassicin
	4-Hydroxyindol-3-ylmethylglucosinolate	4-Hydroxyglucobrassicin

FIGURE 4.1. Structure of major glucosinolates present in canola (Shahidi,1990).

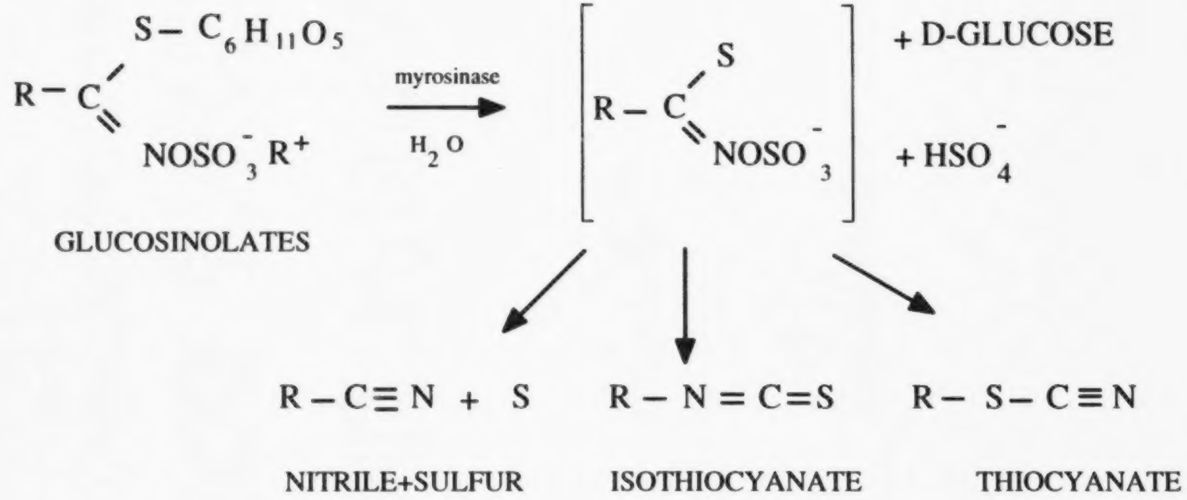


FIGURE 4.2. Enzymatic hydrolysis of glucosinolates.

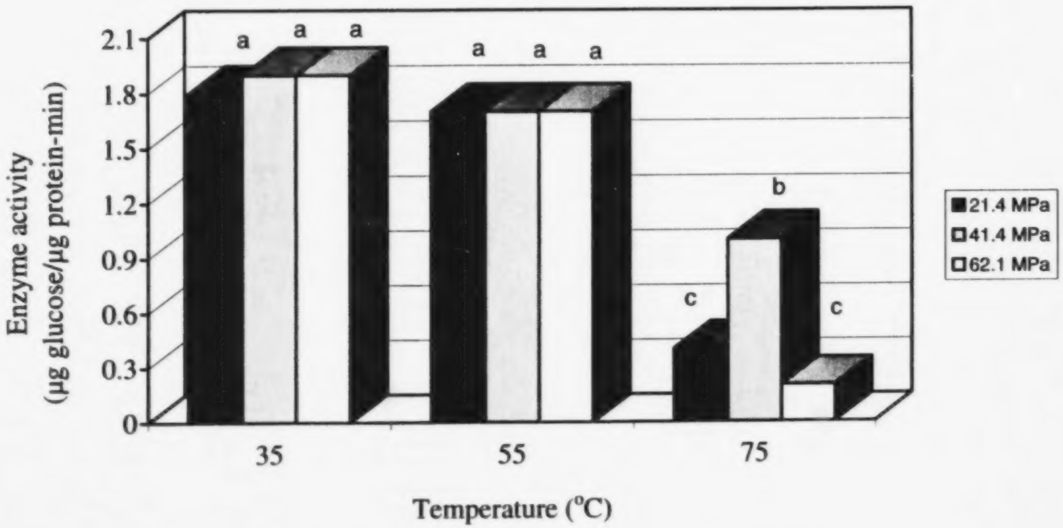


FIGURE 4.3. Effect of pressure and temperature on the myrosinase activity in canola flakes with 19% moisture content. (Bars with the same letter are not significantly different at $p>0.05$ level).

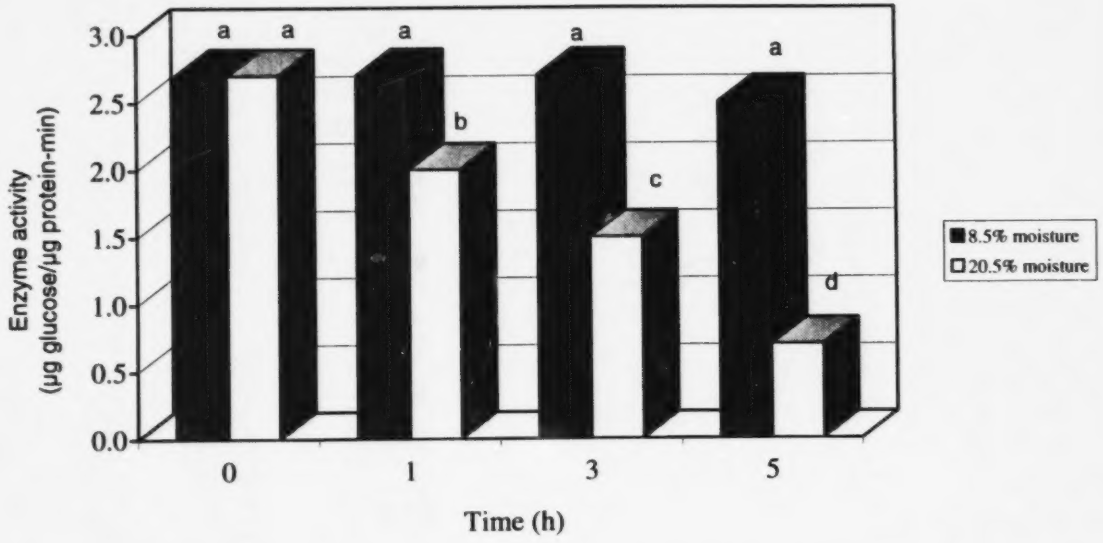


FIGURE 4.4. Effect of time and moisture content of the whole canola seeds on the myrosinase activity during SC-CO_2 treatment at 62.1 MPa and 75°C. (Bars with the same letter are not significantly different $p>0.05$ level).

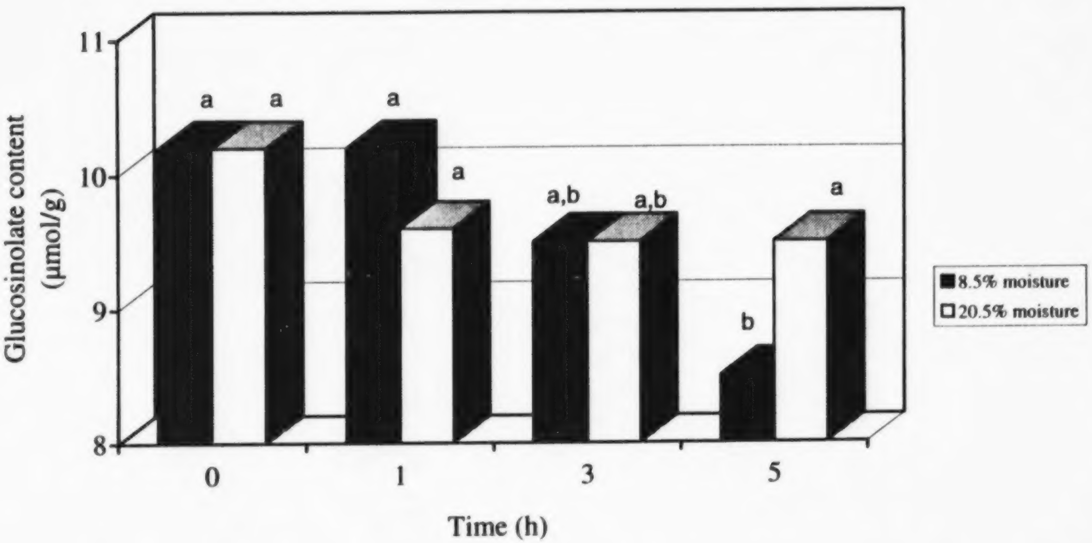


FIGURE 4.5. Effect of time and moisture on the glucosinolate content of whole canola seeds during the SC-CO_2 treatment at 62.1 MPa and 75°C. (Bars with the same letter are not significantly different at $p>0.05$ level).

5. EFFECT OF MOISTURE CONTENT OF ATLANTIC MACKEREL (*Scomber scombrus*) ON SUPERCRITICAL CO_2 EXTRACTION OF OIL AND RESIDUAL PROTEINS[1]

5.1. INTRODUCTION

The use of fish oil for the treatment of diseases goes back to the Middle ages (Stansby, 1990). Over the last decade, scientific studies have shown clearly that increased fish consumption decreases the risk of fatal heart attacks by lowering serum cholesterol levels (Kinsella, 1987). Recent studies on fish oils focus on the effect of ω-3 fatty acids on human health. Effect of dietary fish oils and ω-3 fatty acids on skin disease, hypertension, cancer, multiple sclerosis and rheumatoid arthritis have also been investigated (Karmeli, 1987; Budowski, 1988).

Atlantic mackerel (*Scomber scombrus L.*) belongs to the *Scombridae* family. Canada has one of the largest mackerel stocks in the world. Therefore, it has high economic importance among the various pelagic fish species (Department of Fisheries and Oceans, 1991). Atlantic mackerel is underutilized due to the lack of North American consumer familiarity as well as postharvest handling problems because of its relatively high lipid (1.8-20.6%, depending on the season) and unsaturated long chain fatty acid content (70% of total fatty acids) (Ackman and Eaton, 1971). Currently, meal of underutilized fish species is utilized as animal feed (Bimbo and Crowther, 1992), whereas the demand for proteins as functional food ingredients is expanding (Hardardottir and Kinsella, 1988).

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[1] A version of this chapter is to be submitted to Journal of Food Science for publication.

Conventional fish processing for oil removal from proteins involves cooking, pressing and/or liquid extraction. Since removal of lipids with conventional organic solvents causes protein denaturation and loss of functional properties (Pariser et al., 1978), supercritical fluid extraction (SCFE) of fish muscle might be an alternative technique to produce high quality fish meal and oil. Recently, SCFE has been used for fish processing. The concentration of ω-3 fatty acids in fish oil using supercritical carbon dioxide (SC-CO_2) has been studied extensively (Eisenbach, 1984; Krukonis, 1988; Rizvi et al., 1988; Nilsson et al., 1988, 1989; Higashidate et al., 1990). However, oil extraction directly from high-fat fish muscle has attracted less attention than fish oil fractionation. Extraction of ground freeze-dried Antarctic krill with SC-CO_2 at 25-40 MPa and 40-80°C yielded oils which contained no phospholipids (PL) but mainly triglycerides (TG) with an eicosapentaenoic acid (EPA) content of 11% (Yamaguchi et al., 1986). Ikushima et al. (1986) extracted freeze dried mackerel (*Scomber Japonicus*) powder using SC-CO_2 at 4.9-24.5 MPa and 40°C. It was shown that oil yield increased with pressure and yield was comparable to that of the hot hexane extraction (5 h soxhlet). Also, a kinetic model was developed to express transport phenomena within the solids during the SCFE process. SC-CO_2 and a SC-CO_2/ethanol (EtOH) mixture were used to remove lipids from trout muscle with a moisture content of 74.9 % (w/w)

(Hardardottir and Kinsella, 1988). Increasing pressure and temperature in the ranges of 13.8-34.5 MPa and 40-50°C, respectively, did not improve lipid extraction significantly, most probably due to the very high moisture content of the feed. Increasing extraction times from 3 to 9 h increased oil removal from trout muscle. It was possible to extract 78-97% and 97-99% of the lipids and cholesterol, respectively. The SC-CO_2/EtOH mixture was more effective in removing oil than EtOH-free SC-CO_2. The moisture content of the SC-CO_2 extracted muscle was substantially lower than the initial moisture content, 13.1 and 74.9%, respectively, indicating moisture removal together with lipids (Hardardottir and Kinsella, 1988). More recently, Temelli et al. (1995a, 1995b) investigated SC-CO_2 extraction of freeze-dried Atlantic mackerel *(Scomber scombrus)* emphasizing the changes in proteins due to high pressure oil extraction. It was found that 34.5 MPa and 35°C were the optimum oil removal conditions with minimal changes in residual proteins. Water binding potential and pH of the residual proteins, changes in sarcoplasmic proteins and ω-3 fatty acid composition of the SC-CO_2 extracted oil were reported.

In all but one of the above mentioned studies (Yamaguchi et al., 1986; Ikushima et al., 1986; Temelli et al., 1995a, 1995b), fish samples were freeze dried prior to SC-CO_2 extraction of oil to improve extraction efficiency, whereas Hardardottir and Kinsella (1988) froze trout muscle pieces in liquid nitrogen followed by grinding to increase surface area. Moisture content of the feed materials might interfere with the SC-CO_2 extraction of desired components from the sample matrix. This phenomenon was shown to be very important during the SC-CO_2 extraction of lipids from muscle samples. Extraction of intact muscle presents difficulties due to its fibrous structure and high moisture content (Wehling et al., 1992). King et al. (1989) reported higher extraction rates for meat products which were comminuted and dehydrated prior to SC-CO_2 extraction of lipids. However, optimization of the sample moisture content for SC-CO_2 lipid extraction from muscle tissues and its effect on proteins have not been reported. In addition, correlation of moisture effects to water activity of biological samples should be investigated. Water activity is an indication of the level of free and bound water in the food. The level of free water determines the extent of chemical and enzymatic reactions and microbial growth in the food. Thus, water activity should be a critical factor affecting all component interactions during SC-CO_2 extraction.

The objectives of this study were: a) to study the effect of the moisture content of Atlantic mackerel on the SC-CO_2 extractability of oil, b) to characterize the lipid composition of SC-CO_2 extracts and residual oil in the muscle, c) to analyze changes in sarcoplasmic proteins due to SC-CO_2 extraction at different moisture contents using capillary electrophoresis (CE) and d) to examine the relation between water activity and SC-CO_2 extractability of lipids.

5.2. MATERIALS AND METHODS

Sample preparation

Atlantic mackerel (*Scomber scombrus*) was obtained from Nova Scotia, Canada, during the fall of 1994. The iced fish samples were cleaned, skin-on filleted, blast-frozen at -40°C, and packed in 4.5 kg plastic boxes at the processing plant prior to direct air shipment to Edmonton, AB. The fish were stored at -80°C until they were used. Three batches of fish samples were freeze-dried to moisture contents of 3.8, 10.2, and 26.0% (w/w) under 1 torr vacuum in a REPP freeze-drier (Model FFD-42-WS, Virtis Co. Inc., Gardiner, NY). Then, the fillets were skinned and chopped (Handy ultra chopper, Model HMP3-04, Black and Decker Inc., Brockville, ON) for 15 seconds prior to SC-CO_2 extraction. Moisture content of the samples were determined just before and after each extraction run at 103°C by overnight drying (Woyewoda et al., 1986).

Supercritical CO_2 extraction

A laboratory scale SFE unit (Newport Scientific Inc., Jessup, MD) as described in Chapter 2, was used for oil removal from fish samples. Twenty g of chopped mackerel sample were loaded into the extraction cell. SC-CO_2 extractions of mackerel samples with moisture content of 3.8, 10.2, 26.0 and 64.0% (wet weight basis) were carried out at 35°C and 34.5 MPa according to Temelli et al. (1995a). CO_2 flow rate was 2.2±0.4 g/min. Extraction time was 5 h. The quantities of extract and residual proteins were determined gravimetrically. Protein residue and lipid extracts were stored at -80°C for two weeks before further analysis.

Lipid analysis

Lipid content of the fish samples was determined before and after the SC-CO_2 extraction. Chloroform/methanol mixture was used for lipid extraction according to Woyewoda et al. (1986). Separation of lipid classes and quantitation based on peak areas were carried out by an Iatroscan TH-10 Mark II analyzer equipped with precoated silica gel chromarods SIII (T.M.A. Scientific Supply, Mississauga, ON). The chromarods were developed in a solvent system of benzene:chloroform:methanol:acetic acid (68:8:0.2:0.3, v/v/v) and scanned under the same conditions as given in Chapter 2. Lipid standards (Sigma Chemical Co., St. Louis, MO); cholesterol stearate (5-cholesten-3β-yl octadecanoate), tripalmitin (1,2,3 trihexadecanoyl glycerol), heptadecanoic (C17:0), nonadecanoic (C19:0) and stearic (C18:0) acids, cholesterol (5(6)-cholesten-3β-ol), and L-α-phosphatidylcholine (type XVI-E) were used for peak identification. Lipid standards and samples were kept at -80°C until they were analysed.

Water adsorption isotherm

Adsorption isotherm of Atlantic mackerel muscle tissue was determined by exposing 3 g of freeze-dried and chopped fish sample in desiccators containing saturated salt solutions in water; Appendix I, Table I.1 (Karel, 1975). Water activities (a_w) of the saturated salt solutions used were 0.11, 0.23, 0.32, 0.52, 0.67 and 0.86 (Rockland, 1960). Water and anhydrous phosphorus pentoxide were used as is for one and zero water activities, respectively. To avoid mold growth on the samples at higher water activities (0.67, 0.86 and 1), a vial of formalin was placed in the desiccators. The samples were allowed to equilibrate for 17 days at room temperature (~20°C), after which moisture content of the samples were determined. Initial moisture content of the samples was 3.8% (w/w). Two desiccators containing two samples in each were prepared for each water activity. Moisture content of the samples at each water activity was determined as the average of four samples. Monolayer value for Atlantic mackerel was calculated from BET equation (Labuza, 1968):

$$\frac{a_w}{(1-a_w)X} = \frac{1}{X_m C} + \frac{C-1}{X_m C} a_w \qquad (5.1)$$

where; C = constant

X_m = monolayer moisture content, g water/g dry solid

X = equilibrium moisture content, g water/g dry solid.

A plot of the left hand side of equation (5.1) as a function of a_w yielded a straight line in the range of $a_w<0.4$. From the slope and intercept of this line X_m and C were calculated.

Water activity

Water activity of the fish samples were determined before and after the SC-CO_2 extraction. An AquaLab CX-2 (Decagon Devices Inc., Pullman, WA) unit which uses the chilled-mirror dewpoint technique was used for these measurements.

Color

Hunter "L", "a" and "b" values of the mackerel samples was measured by a Hunter color difference meter (HCDM) (Model D25M/L-2, Hunter Associates Laboratory Inc., Fairfax, VA) just before and after the SC-CO_2 extraction.

Protein content

Sarcoplasmic protein content of fish samples were determined before and after the SC-CO_2 extraction. A 3 g mackerel sample was homogenized for 1 min in 30 mL of phosphate buffer saline solution (1.364 g KH_2PO_4, 2.19 g NaCL, 5 mL of 1% NaN_3, pH adjusted to 7.5 and diluted to 500 mL) using a Virtis mixer immersed in an ice bath. Homogenized samples were centrifuged (Model J2-21, Beckman Instruments Inc., Mississauga, ON) for 30 min at 25,400xg at 4°C. The supernatant was analyzed for protein content according to Lowry et al. (1951). The same extracts were used for capillary electrophoresis analysis.

Capillary electrophoresis

Protein extracts were analyzed by a SDS-PAGE capillary electrophoresis (CE) unit (P/ACE System 2100, Beckman Instruments, Inc., Fullerton, CA) equipped with an on-column UV detector which was set at 214 nm. Cathode and anode were on the injection and detection side, respectively. This is referred to as 'reversed polarity', since the negatively charged sodium dodecyl sulphate (SDS)-protein complexes migrate toward anode in the eCAP SDS 14-200 gel buffer filled capillary column. Prior to injection, protein samples were mixed with sample buffer (0.12 M Tris/HCl/1% SDS, pH 6.6), reference standard, orange G dye, 2-mercaptoethanol, which is a disulphide bond cleaving agent, and deionized water followed by boiling the mixture in a water bath at 100°C for 10 min in closed vials. Analyses were carried out at 20°C under 14.1 kV voltage. The samples were injected into the column by pressure for 60 sec. The fused silica coated capillary column was 47 cm long (40 cm to the detector) and had 100 μm ID. Capillary was rinsed with 1.0 N HCl for 6 min followed by rinsing with SDS 14-200 gel buffer for 6 min between each sample injection. The CE system was calibrated using a standard mixture as shown in Figure 5.1. The electropherograms were acquired and stored on a DELL 316 SX expanded computer using System Gold™ Version 7.1 software package (Beckman Instruments, Fullerton, CA). Peak areas were integrated by the same software. CE peak area percentages were reported.

Statistical analysis

All SC-CO_2 extractions and analysis of samples from each extraction were carried out in duplicate in randomized order and means were reported. Analysis of variance of the results was carried out using General Linear Model procedure of SAS Statistical Software, Version 6 (SAS, 1989). Means of the results were compared by LSD (Least Significant Difference) test at $\alpha = 0.05$ level.

5.3. RESULTS AND DISCUSSION

Extract amount

Effect of sample moisture content on the SC-CO_2 extracts and residual proteins were studied at 34.5 MPa and 35°C since previous studies done with Atlantic mackerel had shown that the highest ω-3 fatty acid concentration in the extract with minimal damage to the proteins was attained under these conditions (Temelli et al., 1995a, 1995b). Material balance calculations as a function of feed moisture content are presented in Figure 5.2. Table 5.1 shows the extract amount and its oil and water contents obtained at different feed moisture levels. Material balance calculations were checked against the solids content of the samples. Accuracy of the solid balance was ±0.1 g. Extract amount increased significantly ($p<0.05$) when moisture content of mackerel samples was decreased to 10.2% from their natural moisture content of 64% prior to SC-CO_2 extraction. The amount of extract obtained at 26.0% moisture was similar to that at 64.0% as well as 10.2% moisture. Further dehydration of samples from 10.2% to 3.8% did not improve the SC-CO_2 extract amount significantly ($p>0.05$). Monolayer adsorption value for Atlantic mackerel was calculated to be 3.24 g H_2O/100 g solid from the water sorption isotherm (Figure 5.3) and BET plot. At 3.8% moisture level the samples contain 3.95 g H_2O/100 g solid. Thus, minimal level of free moisture in the fish samples at 3.8% moisture level results in lowering of the swelling effect in the muscle cell leading to lower diffusion rates.

The amount of oil extracted increased from 0.3 g at 64.0% moisture to 2.5 g with a decrease in the moisture content to 26.0% and remained steady within 2.5-2.7 g range with further dehydration. Only 10% of the oil in the feed was extracted at the natural moisture content of mackerel samples (64%, w/w). Such a low oil recovery rate is due to two reasons: a) high moisture content of the sample acts as a barrier to diffusion of SC-CO_2 into the sample matrix as well as diffusion of oil out of the matrix, thus, reducing SC-CO_2-sample contact, and b) the pasty consistency of chopped muscle samples also reduces SC-CO_2-sample contact in the extractor cell. Indeed, when chicken muscle chunks were used as feed material for SC-CO_2 extraction oil removal efficiency was higher than that of the chopped samples (Froning et al., 1994).

These results indicate that it is not necessary to dry Atlantic mackerel samples beyond ~26.0% moisture to achieve higher oil yields during SC-CO_2 extraction. This would also result in savings in time (since freeze drying is a very slow process) and energy. Furthermore, shorter drying times would reduce the risk of quality deterioration during processing.

Moisture

The moisture content of the feed material and residual meal after SC-CO_2 extraction was determined to facilitate material balance calculations (Figure 5.2 and Table 5.1). Material balance calculations showed that some water was co-extracted along with oil during the SC-CO_2 extraction of fish samples. Similar results were obtained with canola flakes as discussed in Chapter 3, other oil seeds (Von Eggers and Stein, 1984; Snyder et al., 1984) and chicken meat (Chao et al., 1991). Highest moisture loss from the samples was attained at the highest moisture level studied which was 1.8 g at 64% moisture level (Table 5.1). Moisture loss during SC-CO_2 extraction of dehydrated fish samples was in the range of 0.3-0.6 g; these results were not statistically different ($p>0.05$). It was not possible to determine the amount of water in the SC-CO_2 extracted oil experimentally due to the small size of the extracts. However, it was observed that extracts obtained at 64% moisture level consisted mainly of milky water droplets. This observation was supported by the material balance calculations, since oil made up only 15.0% of the extracts at that moisture level. Dehydration of mackerel samples down to 26% moisture level resulted in a significant drop ($p<0.05$) in the amount of water co-extracted along with oil (Table 5.1). In addition to the low extraction efficiency as discussed above, conducting SC-CO_2 oil extraction at high moisture content has the disadvantage of having large amounts of water in the oil extract which needs to be separated.

Water activity of the residual meal after SC-CO_2 extraction was slightly lower than that of the feed material (Table 5.2). Although the largest amount of water was extracted from the samples at 64% ($a_w = 0.997$) moisture level, the largest drop in water activity was at 10.2% ($a_w = 0.718$) moisture level. This can be explained with the water adsorption isotherm of Atlantic mackerel (Figure 5.3). At $a_w > 0.8$ region, slope of moisture content vs a_w curve is much higher than that of the lower a_w region, indicating that a larger change in moisture content of the samples is required for a given a_w

change. It was shown that a large water fraction in proportion to the amount of oil was obtained towards the end of the SC-CO_2 oilseed extraction (Von Eggers and Stein, 1984; Snyder et al., 1984). In our experiments, extraction time was limited to 5 h. If the SC-CO_2 extractions were continued till completion, decrease in the water activity of the samples should become more significant. This might be a positive attribute for residual proteins during storage since enzyme reactions tend to slow down and product becomes more stable at lower water activity levels.

Lipid composition

Both SC-CO_2 extracts and chloroform/methanol extracts of feed and residual meal consisted mainly of TG's (Table 5.3). TG content of oil in the feed was slightly higher than that of the residual meal. However, PL content of the oil in the residual meal was considerably higher than that of the feed material. Increased concentration of PL in the residue is due to the higher selectivity of nonpolar CO_2 towards neutral TG. These results correlate well with the composition of the SC-CO_2 extracts where TG concentration was higher and PL concentration was much lower than that of the meal and feed material. SC-CO_2 extracted oil had slightly higher PL concentration at higher moisture levels. Similar results were obtained with SC-CO_2 extracted cardamom seeds (Gopalakrishnan and Narayanan, 1991). Higher concentration of polar compounds in the SC-CO_2 extracts at higher moisture levels is attributed to the cosolvent effect of water, where the presence of polar water in the supercritical phase modifies the polarity of supercritical solvent (Brunner, 1983). FFA acid contents of SC-CO_2 extracts were higher than that of the meal and feed material except at the natural moisture content of fish samples. At that moisture level, FFA concentration of SC-CO_2 extracts and residual meal were similar.

Color

Hunter color values of the samples were measured before and after extraction to assess the effect of SC-CO_2 treatment on the color of the residual proteins (Table 5.4). Hunter "L", "a", "b" values describe lightness, redness-greenness, and yellowness-blueness of the samples, respectively. SC-CO_2 extracted mackerel samples had higher "L", "b" and lower "a" values compared to the feed materials which indicate that SC-CO_2 produces lighter colored products with less redness. Similar results were reported for chicken and beef samples (Wehling et al., 1992; Froning et al., 1994). Effect of SC-CO_2 on the color of residual meal was the largest at 26% moisture level, where the residual meal was much lighter than the feed material. Lighter color of the SC-CO_2 extracted meat samples was attributed to the extraction of pigments with SC-CO_2 (Wehling et al., 1992). As Wehling et al. (1992) also indicated, a product with lighter color would be more desirable as a protein source in various prepared foods.

Protein

Fish samples were analyzed for their sarcoplasmic protein content before and after the SC-CO_2 extraction (Fig. 5.4). All of the residue samples had significantly lower ($p<0.05$) lower protein content, as measured by Lowry et al. (1951) method, compared to feed samples. The largest decrease in protein content was observed at 26.0% moisture level. These results indicate that there were changes taking place in the structure of the sarcoplasmic proteins during the SC-CO_2 extraction so that they can not be measured by the Lowry et al. (1951) method. The same trend was observed in an other study by Temelli et al. (1995a) with Atlantic mackerel.

Capillary electrophoresis technique was used to analyze the changes in the sarcoplasmic proteins. An electropherogram of the sarcoplasmic protein extracts of mackerel sample with 10.2% moisture is given in Figure 5.5, which was typical for all the samples analyzed. Figures 5.6-5.8 summarize the changes in the sarcoplasmic proteins as a function of sample moisture content before and after SC-CO_2 extraction. Proteins were classified into three groups based on their molecular weight (MW) as follows: a) MW <50 kDa (Fig. 5.6), b) 50<MW<100 kDa (Fig. 5.7), and c) MW>100 kDa (Fig. 5.8). CE area percentage of proteins with MW<50 kDa were slightly lower following extraction for all samples compared to the feed samples, except at 64% moisture level (Fig. 5.6). A similar trend was observed for proteins with MW 50-100 kDa at all moisture levels; however, the decrease following extraction was quite substantial (Fig. 5.7). For example, at 26% moisture level, proteins with MW 50-100 kDa completely diminished. On the other hand, CE area percentage of proteins with MW>100 kDa were all higher following the extraction (Fig. 5.8). These results indicate aggregation of low molecular weight proteins, especially those with a MW of 50-100 kDa, to form larger proteins of MW>100 kDa during high pressure extraction. Such changes in proteins should in part be responsible for the decrease in the protein content as measured by the Lowry et al. (1951) method (Fig. 5.4). Similar results were reported by Yamamoto et al. (1992) and Temelli et al. (1995b). Contraction of beef muscle sarcomeres was observed by Suzuki et al. (1992) using scanning electron microscopy following a high hydrostatic pressure treatment (150 MPa, 5 min). Although the pressure used by Suzuki et al. (1992) was much higher than that of our study, contraction of sarcomeres under pressure might be forcing sarcoplasmic proteins to aggregate even at lower pressures. However, contraction behaviour of sarcorneres with pressure needs to be further examined. Mackerel samples with 10.2% moisture content seem to be the least affected in terms of structural changes in proteins occuring during SC-CO_2 extraction.

5.4. CONCLUSIONS

To achieve high oil recovery from the fish muscle with SC-CO_2, dehydration of feed down to 26.0% appears to be sufficient, since further dehydration did not improve extraction efficiency. Furthermore, at this moisture level obtaining minimal moisture in the SC-CO_2 extracted samples is a positive attribute. However, if residual meal is to be used as a protein concentrate, dehydration down to 10.2% would be necessary without sacrificing oil extraction yield, because sarcoplasmic proteins appear to be the least affected during the SC-CO_2 extraction at 10.2% moisture. Lighter color of SC-CO_2 extracted fish samples should be a desirable attribute as an ingredient for potential food applications. Higher TG and lower PL content of SC-CO_2 extracted fish oil would lower further refining costs. Sensory and functional properties of residual proteins in the fish samples after SC-CO_2 extraction should be consired for further research. SC-CO_2 extraction of fish muscle might be an alternative technique to conventional fish processing techniques to obtain high quality value-added products.

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TABLE 5.1. Effect of feed material moisture content on the total SC-CO_2 extract amount and its moisture and oil contents as calculated from material balance for 20 g feed material at 34.5 MPa and 35°C

Feed moisture content (%, w/w)	Total extract amount (g)	Amount of water extracted (g)	Amount of oil extracted (g)
64.0	2.0[a]	1.8[a]	0.3
26.0	2.8[ab]	0.3[b]	2.5
10.2	3.4[b]	0.6[b]	2.7
3.8	3.2[b]	0.5[b]	2.6

[a,b]Means with the same letter in each column are not significantly different at $p>0.05$ level.

TABLE 5.2. Effect of SC-CO_2 extraction on the water activity of residual meal.

Feed moisture content (%, w/w)	Before extraction	After extraction
64.0	0.997	0.991
26.0	0.940	0.930
10.2	0.718	0.608
3.8	0.242	0.181

TABLE 5.3. Lipid composition of oil in the feed materials, residual meals and SC-CO_2 extracts at various sample moisture content.

Feed moisture content (%, w/w)	Sample	TG[1]	FFA[1]	PL[1]
64.0	Oil in feed	97.9	0.3	1.5
	Oil in meal	95.6	0.5	3.2
	SC-CO_2 extract	98.7	0.5	0.6
26.0	Oil in feed	96.9	0.6	1.7
	Oil in meal	92.2	0.9	6.0
	SC-CO_2 extract	96.7	1.5	0.9
10.2	Oil in feed	96.5	0.5	2.3
	Oil in meal	92.9	0.6	5.7
	SC-CO_2 extract	98.3	1.0	0.3
3.8	Oil in feed	96.1	0.6	2.7
	Oil in meal	95.2	0.5	3.6
	SC-CO_2 extract	96.9	1.9	0.3

[1] Iatroscan area percentages

TABLE 5.4. Effect of SC-CO_2 extraction on the color of residual meal.

Feed moisture content (%, w/w)	Sample	L	a	b
64.0	Before extraction	47.7	2.0	11.9
	After extraction	49.4	0.5	12.5
26.0	Before extraction	42.8	3.9	13.3
	After extraction	52.0	1.4	16.9
10.2	Before extraction	52.0	5.0	14.1
	After extraction	57.3	2.2	15.9
3.8	Before extraction	54.2	5.0	14.4
	After extraction	57.8	3.6	15.0

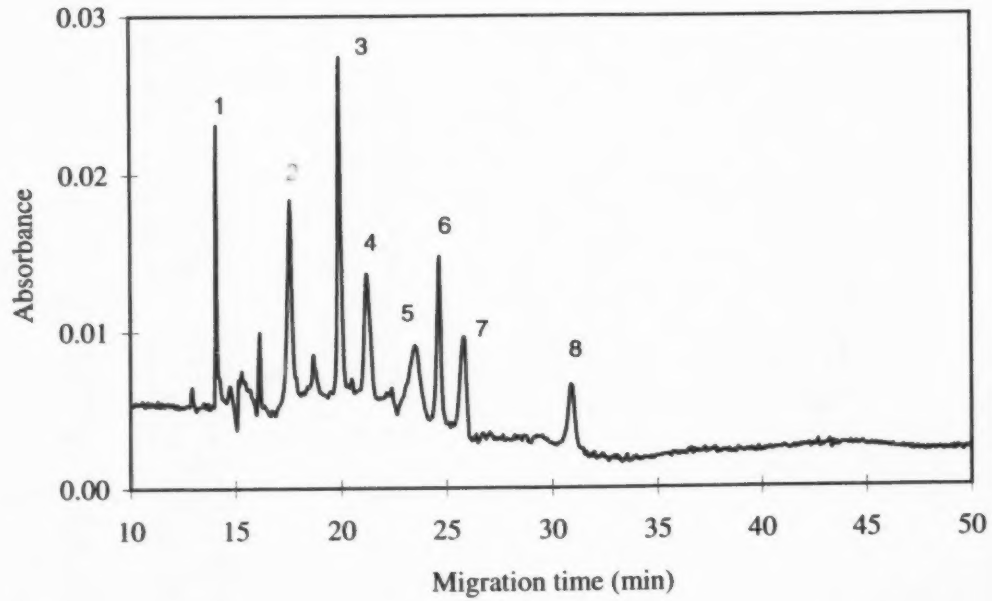

FIGURE 5.1. Electropherogram of standard mixture: (1) Orange G, (2) α-Lactalbumin (14.2 kDa), (3) Carbonic anhydrase (29 kDa), (4) Ovalbumin (45 kDa), (5) Bovine serum albumin (66 kDa), (6) Phosphorylase b (97.4 kDa), (7) β-galactosidase (116 kDa), (8) Myosin (205 kDa).

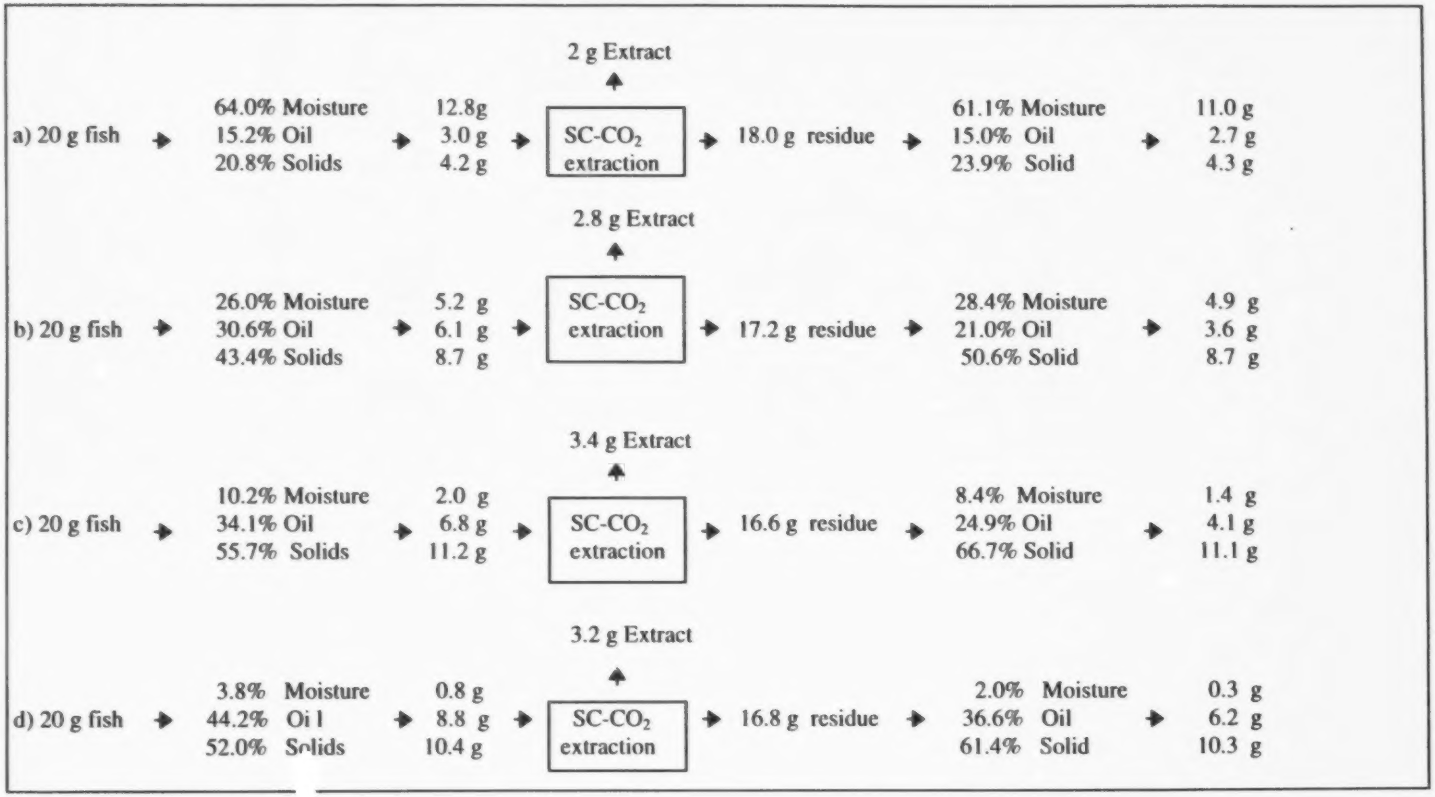

FIGURE 5.2. Material balance calculations.

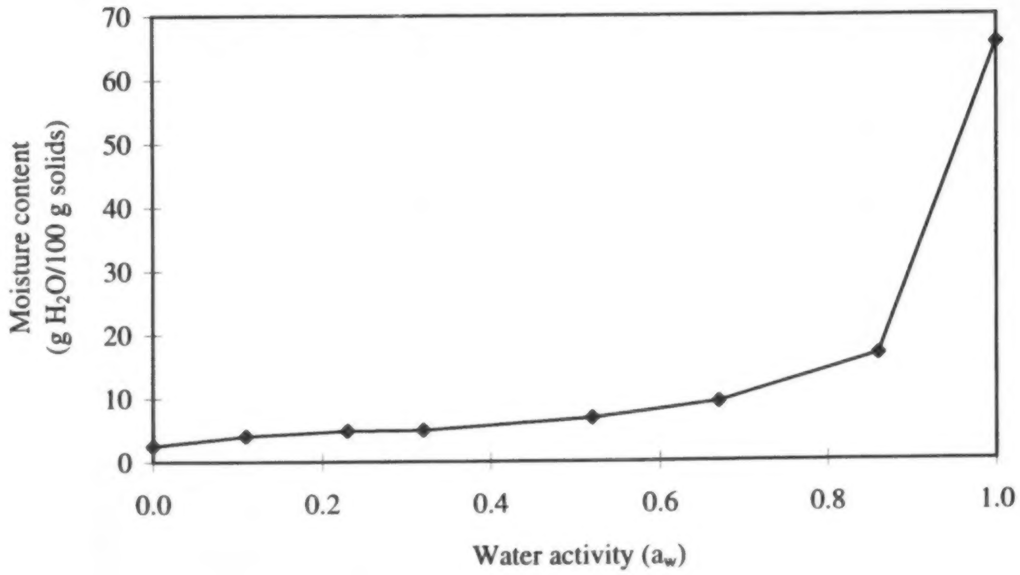

FIGURE 5.3. Water adsorption isotherm of Atlantic mackerel.

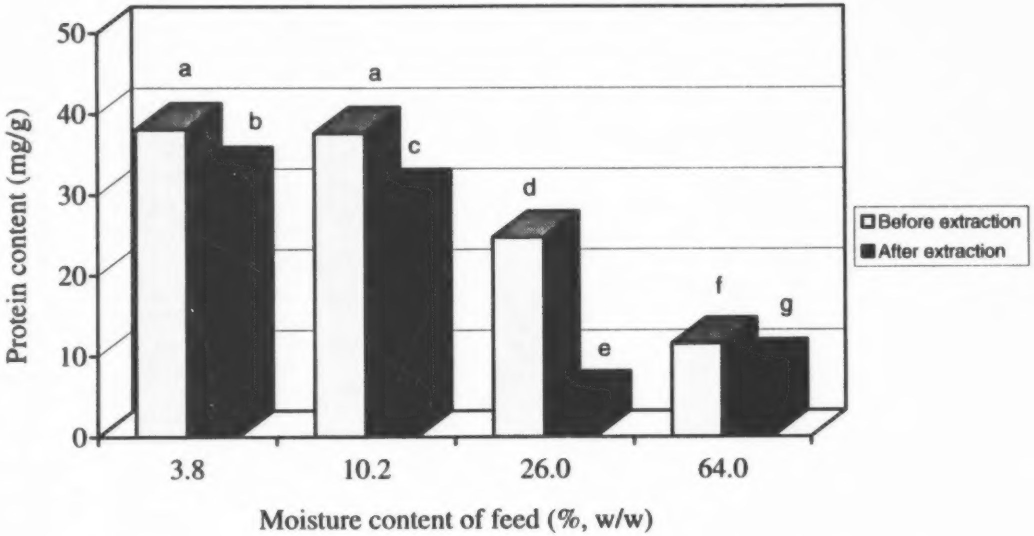

FIGURE 5.4. Protein content of the sarcoplasmic extracts of mackerel samples before and after the SC-CO_2 extraction at 34.5MPa and 35°C for 5 h. (Bars with the same letter are not significantly different at $p>0.05$ level).

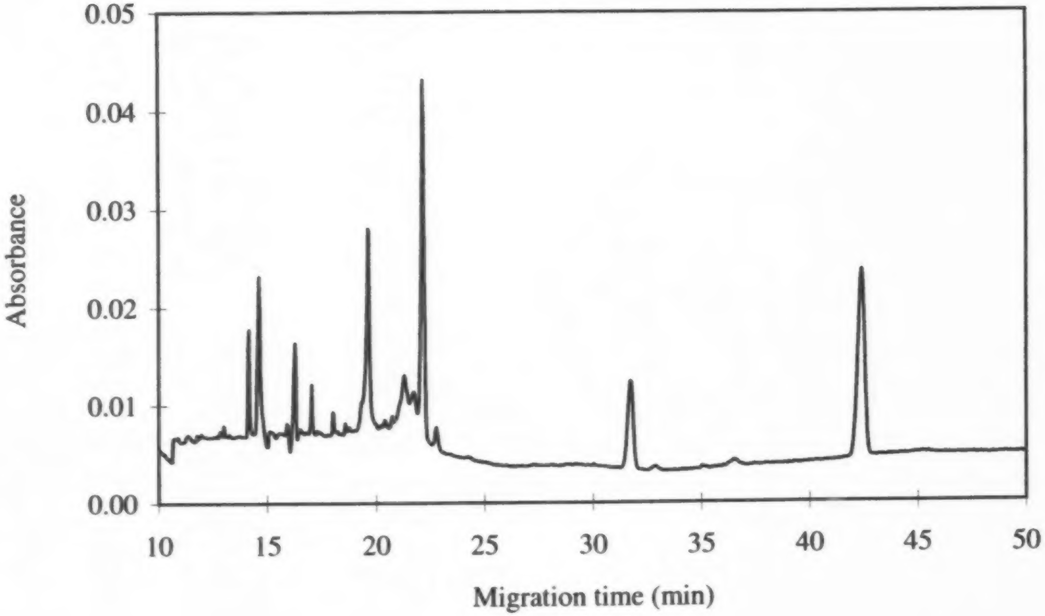

FIGURE 5.5. Electropherogram of Atlantic mackerel sarcoplasmic protein extracts at 10.2% (w/w) moisture level before SC-CO_2 extraction.

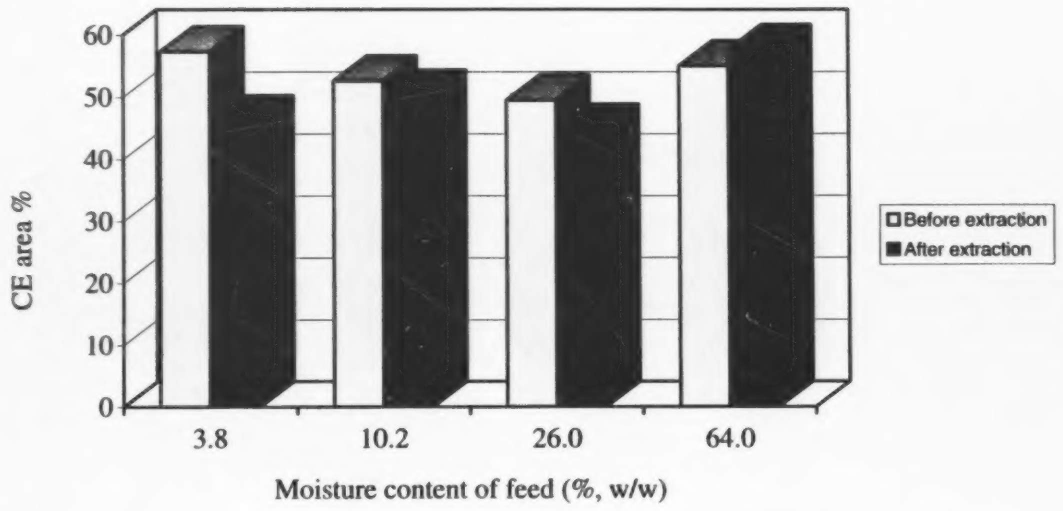

FIGURE 5.6. CE area% of MW<50 kDa sarcoplasmic proteins in Atlantic mackerel before and after the SC-CO_2 extraction at 34.5 MPa and 35°C for 5 h.

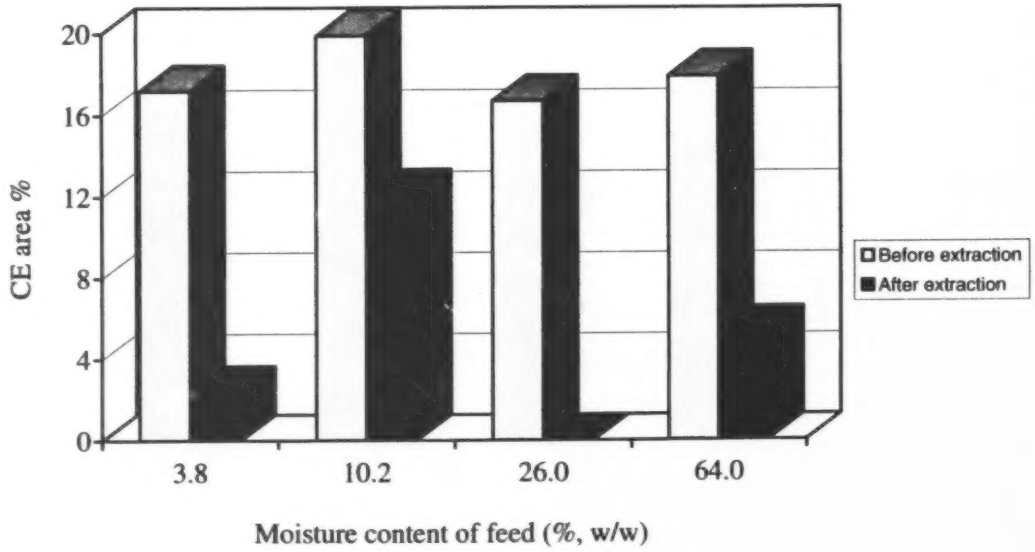

FIGURE 5.7. CE area % of MW 50-100 kDa proteins in Atlantic mackerel before and after the SC-CO_2 extraction at 34.5 MPa and 35°C for 5 h.

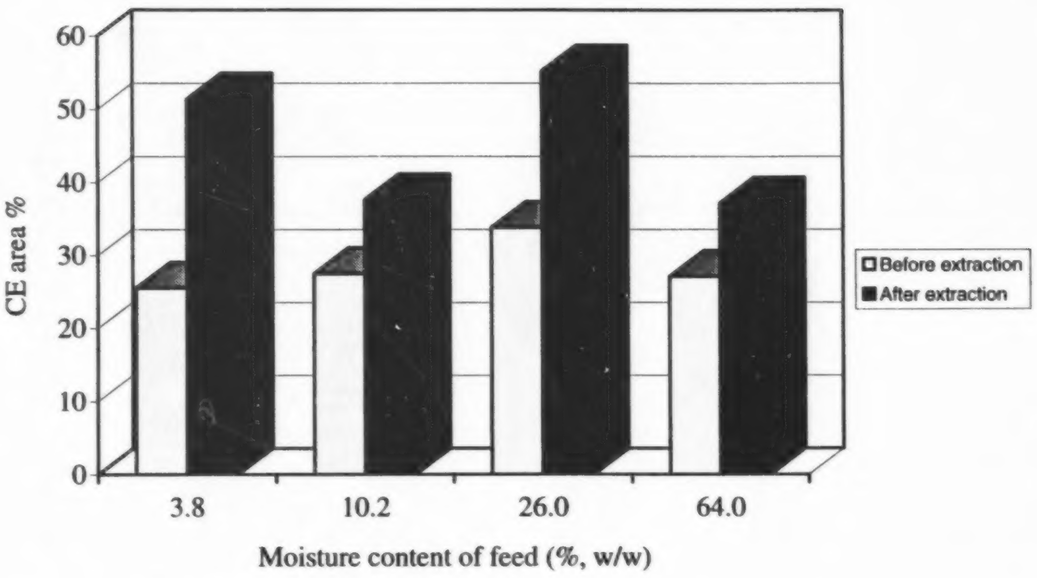

FIGURE 5.8. CE area % of MW>100 kDa proteins in Atlantic mackerel before and after the SC-CO_2 extraction at 34.5 MPa and 35°C for 5 h.

6. CONCLUSIONS AND RECOMMENDATIONS

Edible oils from oilseeds are commercially extracted with hexane in a semi-continuous countercurrent process. SC-CO_2 is an alternative solvent for various food process applications, since it is non-toxic, non-explosive, cheap, readily available and easily removable from the product. Although SC-CO_2 extraction of edible oils from various plant and animal sources has been studied extensively (Friedrich, 1984; Bulley et al., 1984; Ikushima et al., 1986; Fattori et al., 1987; Hardardottir and Kinsella, 1988; Temelli, 1992), optimization of all processing parameters and evaluation of products need further research. Furthermore, our understanding of the interactions between neutral lipids/polar lipids/water/solid matrix/SC-CO_2/cosolvent components during this high pressure extraction process is limited. Thus, the main objectives of this thesis were to extract and fractionate phospholipids (PL) from canola using ethanol (EtOH) as a cosolvent and to examine the effect of various processing parameters, such as temperature, pressure and moisture content of feed material on extract composition, residual proteins and enzyme activity during SC-CO_2 extraction of edible oils from plant and animal sources. Canola and Atlantic mackerel (*Scomber scombrus*) were chosen as edible oil sources from plant and animal origin, respectively, because of their importance to the Canadian economy. Canola and Atlantic mackerel also represent products with low and high water activity levels, respectively, presenting different challenges in lipid extractability with SC-CO_2.

It was shown that SC-CO_2 did not extract relatively polar PL (Friedrich and Pryde, 1984; Fattori et al., 1987); therefore, PL are left in the meal during SC-CO_2 extraction of oilseeds. For SC-CO_2 extraction of oilseeds to be commercialized, recovery of all valuable components is essential for maximum utilization of natural products and maximum profitability. In Chapter 2, SC-CO_2/EtOH mixture was used to extract and fractionate PL from canola flakes, meal and acetone insolubles (AI). Extraction of PL directly from full fat canola flakes was not possible even in the presence of ~10% EtOH in SC-CO_2. However, PL could be recovered in a second step with SC-CO_2/EtOH mixture after triglycerides (TG) were extracted with EtOH-free SC-CO_2. It was concluded that maximum TG removal in the first step would increase PL extraction efficiency and minimize oil in the PL extracts. Soaking of feed material with EtOH was a new approach to increase PL recovery and fractionation. Soaking of canola meal with EtOH improved PL recovery considerably. It may be possible to scale up this treatment by spraying ethanol on to the bed of flakes to saturate them with ethanol following EtOH-free SC-CO_2 extraction of oil and then extracting PL with SC-CO_2/EtOH. Feasibility of such a soaking treatment at larger scales needs to be further evaluated since extraction time and cost will increase while improving yield. Obviously, the soaking time between the two extraction steps needs to be minimized. When AI were the feed material, soaking prior to extraction did not improve PL recovery due to cake formation in the extractor in the presence of liquid EtOH. Caking problems have to be solved before any further investigation of optimum conditions for SC-CO_2/EtOH extraction of AI. Design of a SCFE unit with a mixing mechanism in the extractor for better contact between solvent and the feed material should improve extraction efficiency.

The recovery of valuable PL with SC-CO_2/EtOH mixture following extraction of oil with EtOH-free SC-CO_2 and the potential for obtaining PC-enriched phospholipids as demonstrated in this study should improve the feasibility of the SCFE of oilseeds. The high price of lecithin, 2-10 times the price of soybean (Szuhaj, 1983) and even the higher price of PC enriched lecithins (up to $1000/kg) indicate the need for their recovery for favorable process economics. In Canada, 60% of the annual canola production is not processed. Such an important agricultural commodity should be processed into high-value products (oil, meal, lecithin) and SCFE should be considered as an alternative to hexane extraction for future investments. This will certainly require detailed economic feasibility comparison for the two process.

Presence of water in biological feed materials is an important factor affecting the SC-CO_2 extractability of desired compounds from a solid matrix. As well, mass transfer kinetics of extraction is affected by the moisture level. Thermodynamic modelling of numerous pure solute/SC-CO_2/water systems have been studied, however, extrapolation of these model system results to natural food and other biological systems is difficult due to their complex nature. Therefore, every system has to be studied individually. In Chapter 3, the effect of processing parameters such as temperature, pressure, moisture content and heat pre-treatment of canola flakes on the lipid composition of SC-CO_2

extracts were studied. When cooked and preheated canola flakes were extracted with $SC\text{-}CO_2$, extract yield increased ($p<0.05$) with pressure. The same trend was observed with temperature ($p<0.05$) at high pressures; however, at 20.7 MPa, extract yield decreased with temperature due to crossover of solubility isotherms. Color of the $SC\text{-}CO_2$ extracts from preheated samples were darker than that of the cooked samples. Extracts obtained at low pressure and high temperature were turbid and thicker than the extracts obtained at high pressure and low temperature for both cooked and preheated samples. It was shown that some water was co-extracted with the oil; therefore, it is essential to determine the amount of water in the $SC\text{-}CO_2$ extracts for the optimization of oil yield. Addition of a second collection unit to separate water from the oil extract would be beneficial. Although moisture modification of the feed material increased the extract yield from preheated samples slightly, the effect of moisture on the amount of extract was not significant ($p>0.05$). Amount of water coextracted along with oil increased with increasing moisture content of the feed. Free fatty acid (FFA) content of the $SC\text{-}CO_2$ extracts increased slightly for preheated and decreased for cooked canola samples with an increase in the initial moisture level. Although the cooking process improved the extract amount only slightly it resulted in significantly ($p<0.05$) higher FFA levels in $SC\text{-}CO_2$ extracts compared to extracts of the preheated samples. The effect of extraction conditions on the amount and lipid composition of $SC\text{-}CO_2$ extracts of canola flakes was more pronounced than the seed pre-treatments studied. Therefore, for higher oil yield and minimal FFA and moisture contents in the oil and lighter color products, canola flakes should be extracted at higher pressures and ~10% (w/w, as is basis) moisture levels. Further characterization including polar lipid composition, keeping quality, nonsaponifiable content and sensory properties of the SC-CO_2 canola extracts obtained at different extraction conditions and the effect of the degree of extraction on the lipid composition should be considered for further research.

In Chapter 4, effect of SC-CO_2 extraction conditions such as temperature, pressure and feed moisture content on the endogenous canola enzyme, myrosinase, was studied. Extent of glucosinolate degradation in flaked and whole canola seeds under SC-CO_2 extraction conditions were also examined. It was shown that myrosinase inactivation and glucosinolate degradation was minimal under SC-CO_2 conditions at low moisture levels in flakes which were not cooked. However, in industrial scale operations, the endogenous enzyme myrosinase has to be inactivated prior to SC-CO_2 extraction since some glucosinolate degradation is inevitable during flaking, seed and meal handling. Although modification of feed moisture content prior to SC-CO_2 treatment resulted in more efficient myrosinase inactivation, such moisture modification is not recommended since it also accelerates glucosinolate hydrolysis and toxic hydrolysis products might be extracted along with oil. These compounds are not desired in the meal either, since it is to be used as animal feed.

In Chapter 5, the effect of moisture content of Atlantic mackerel on oil extraction efficiency and residual proteins was examined during SC-CO_2 extraction. This study was carried out at 35.4 MPa and 35°C, since previous studies done with Atlantic mackerel had shown that highest ω-3 fatty acid concentration in the extract with minimal damage to the proteins was attained under these conditions (Temelli et al., 1995a, 1995b). Lipid composition of SC-CO_2 extracts and residual oil in the muscle were characterized. It was found that dehydration of fish muscle samples down to 26% moisture level was adequate for maximum oil removal, since further dehydration did not improve oil extraction efficiency. This would result in savings in energy needed for drying. Furthermore, shorter drying times would reduce the risk of quality deterioration during processing. However, if residual meal is to be used as a protein concentrate, further dehydration down to 10.2% might be advantageous, since sarcoplasmic proteins seem to be the least affected during SC-CO_2 extraction at this moisture level. Also, aggregation of low molecular weight (MW) sarcoplasmic proteins, especially those with a MW of 50-100 kDa, to form larger proteins of MW>100 kDa was observed during SC-CO_2 extraction of mackerel at all moisture levels studied. BET monolayer value of Atlantic mackerel was calculated to be 3.24 g H_2O/100 g solid. Dehydration of feed material down to about BET values did not improve oil recovery significantly ($p>0.05$) compared to the other dehydrated samples, most probably due to the excessive shrinkage of cell structure reducing diffusion rates in the sample matrix. SC-CO_2 extracted fish muscle had lighter color than the feed material, which should be a desirable attribute for its potential use as an ingredient for food applications. SC-CO_2 extracts had higher TG and lower PL content than the oil residue in the fish samples. Lower PL content of the fish oil would lower its further refining costs. SC-CO_2 extraction of fish muscle might be an alternative technique to conventional fish processing techniques. However, functional properties of residual proteins, sensory evaluation of SC-CO_2 extracted fish oil and residual proteins, incorporation of protein residue into product formulations such as surimi and evaluation of their performance should be considered for further research. Better understanding of the changes in proteins under SC-CO_2 conditions is needed for development of high quality, value-added products. The economic feasibility of freeze-drying combined with SCFE is questionable. However, the resulting high quality products may find applications in specialty food and pharmaceutical areas. On the other hand, dehydration techniques other than freeze-drying should be given consideration.

In conclusion, this thesis demonstrated the effect of the presence of a third component, such as ethanol and/or water, in the SC-CO_2 phase on the lipid extraction efficiency, composition and color of extracts and residual proteins of plant and animal origin. However, our understanding of the complex interactions between different components in a high pressure SC-CO_2 environment is limited. Design of optimum separation processes and equipment requires knowledge of mass transfer kinetics as well as phase behaviour. Mass transfer modelling of SC-CO_2 extraction of lipids and essential oils have been reported (Bulley et al., 1984; Goto et al., 1993; Roy et al., 1994), but these studies do not address the complexities of the presence of water or addition of ethanol into SC-CO_2. Moreover, phase diagrams for such complex multicomponent systems are not available. Better understanding of SC-CO_2/ethanol/water/lipids/solid matrix interactions at the molecular level and its mathematical modelling is essential for design and optimization of industrial scale applications. In this thesis, the use of water activity as a parameter to study oil extractability was a new approach in an effort to understand some of these interactions.The effect of water is especially important for all food applications since water is a part of biological materials and its removal requires additional processing prior to SC-CO_2 extraction adding to the processing cost and time, and most of the time leading to quality deterioration.

6.1. REFERENCES

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7. APPENDIX I

TABLE I.1: Saturated salt solutions used for achieving a constant water activity (Rockland, 1960).

Name	Formula	Water Activity
Phosphorous pentoxide[1]	P_2O_5	0.00
Lithium chloride	$LiCl_2.H_2O$	0.11
Potassim acetate	CH_3COOK	0.23
Calcium chloride	$CaCl_2$	0.32
Magnesium nitrate	$Mg(NO_3)_2$	0.52
Cupric chloride	$CuCl_2$	0.67
Potassium chloride	KCl	0.86
Water	H_2O	1.00

[1] used as is